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14. ABSTRACT

Brain tissue is highly heterogeneous with different functions localized in specific areas. Free radicals are generated endogenously by the mitochondria in the cell. Mitochondrial electron transport chain (ETC) with the help of five ETC complexes is involved in the generation of free radicals and ATP (energy). We compared the levels of mitochondrial ETC complexes in frozen brain samples from the cerebellum and frontal, temporal, parietal and occipital cortices of autistic and age-matched normal subjects. In autism, a significant deficit in mitochondrial ETC complexes was observed in frontal cortex, temporal cortex, and cerebellum. In contrast, no significant change in ETC complexes was observed in other brain regions between autism and control groups. These results suggest mitochondrial dysfunction in autism, which will lead to oxidative stress and abnormal brain energy metabolism in autism. Autism spectrum disorders (ASDs) are complex neurodevelopmental disorders. The complexity of ASDs is further increased because some affected individuals fall in the subgroup of regressive autism. We report here that individuals with regressive autism have decreased activity and expression of protein kinase A (PKA) in the frontal cortex of the brain. Such changes were not observed in individuals with non-regressive autism. PKA is a cyclic AMP–dependent protein kinase that is involved in cognitive functions and memory formation. These results suggest that abnormal cellular signaling in the frontal lobe of the brain may be associated with regression in autism.

15. SUBJECT TERMS
Oxidative stress in autistic brain, free radicals, mitochondria, energy, protein kinases, cell signaling

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SUBPROJECT 3

Oxidative damage and inflammation in the brains of autistic subjects: Correlation with severity and phenotypes.

PI: Abha Chauhan, Ph.D.

INTRODUCTION

While the cause of autism remains elusive, autism is considered a multifactorial disorder that is influenced by genetic and environmental factors. Accumulating evidence suggests that oxidative stress may provide a link between susceptibility genes and pre- and post-natal environmental risk agents in the pathophysiology of autism [reviewed in 1, 2]. Under normal conditions**,** a dynamic equilibrium exists between the production of free radicals, i.e. reactive oxygen species (ROS) and the anti-oxidant capacity of the cell. These ROS are highly toxic, and if not removed or neutralized, they react with lipids, proteins and nucleic acids and damage membrane properties and cellular functions. Oxidative stress is known to be associated with premature aging of cells and can lead to inflammation, damaged cell membranes, autoimmunity and cell death. The brain is highly vulnerable to oxidative stress due to its limited antioxidant capacity, higher energy requirement and high amounts of unsaturated lipids and iron [3]. The brain makes up about 2% of body mass but consumes 20% of metabolic oxygen. The vast majority of energy is used by the neurons [4].

Extensive evidence suggests the presence of oxidative stress in peripheral tissues in children with autism [1]. We have reported that levels of malonyldialdehyde, a marker of lipid peroxidation, are increased in the plasma from children with autism [5]. Other studies on erythrocytes and urine samples have also indicated increased levels of lipid peroxidation markers in autism, thus confirming an increased oxidative stress in autism [6, 7]. Recent studies from our and other groups with postmortem brain tissues have shown elevated levels of markers of oxidative damage, coupled with reduced antioxidant status in the cerebellum, frontal and temporal cortex of the brain of individuals with autism as compared to age-matched control subjects. We observed brain region-specific increased levels of lipid hydroperoxide [8], a product of fatty acid oxidation; of malonyldialdehyde [9], an end-product of lipid peroxidation; of 8-hydroxy-2$^{'}$-deoxyguanosine (8-OH-dG) [10], a marker of oxidative DNA damage; and of protein carbonyl [11], a marker of protein oxidation in autism. Other groups have also reported elevated expression of carboxyethyl pyrrole [12], a marker of lipid-derived oxidative protein modification, and of 3-nitrotyrosine [13], a marker of protein nitration, in postmortem brain samples from autistic subjects.

Glutathione (GSH) is the most important endogenous antioxidant in human tissues, which neutralizes ROS, and participates in detoxification and elimination of environmental toxins. Due to the lack of glutathione-producing capacity by neurons, the brain has a limited capacity to detoxify ROS. Therefore, neurons are the first cells to be affected by the increase in ROS and shortage of antioxidants and, as a result, they are most susceptible to oxidative stress.
We observed a decrease in GSH, an increase in its oxidized disulfide form (GSSG) and a decrease in the redox ratio of GSH/GSSG in the cerebellum and temporal cortex of individuals with autism, suggesting a glutathione redox imbalance in autism [14]. In the cerebellum and frontal cortex of individuals with autism, we have also reported increased activities of Na^{+}/K^{+}-

ATPase and Ca^{2+} /Mg^{2+}-ATPase, the membrane-bound enzymes, which maintain intracellular gradients of ions that are essential for signal transduction [15].

Mitochondria are the primary source of free radicals, and are central to many cellular functions including the generation of ATP (energy). They also trigger apoptosis, i.e. cell death. Neurons in particular rely on mitochondria because of their high levels of activity and subsequent need for energy. The free radicals are generated endogenously during oxidative metabolism and energy production by mitochondria [16]. Electron transport chain (ETC) in mitochondria is a prime site for free radicals generation. Mitochondria generate ATP by generation of protons gradient (membrane potential) with the help of five ETC complexes: *Complex I* (NADH dehydrogenase), *Complex II* (succinate dehydrogenase), *Complex III* (cytochrome bc_1 complex), *Complex IV* (cytochrome *c* oxidase), and *ATP synthase*, also known as *complex V*. The changes in the mitochondrial ETC have been reported in several neurodegenerative disorders.

Protein kinases are known to play important roles in cellular signaling pathways and are involved in brain development. Protein kinase A (PKA) is a cyclic adenosine monophosphate (cAMP)–dependent protein kinase that is involved in cognitive functions and memory formation [17–19]. Several studies have implicated the role of PKA in neuropsychiatric disorders such as schizophrenia, bipolar affective disorder, obsessive compulsive disorder, and panic disorders.

Brain tissue is highly heterogeneous with specific functions localized in specific areas of brain. In this year, we analysed the status of mitochondrial ETC, and the activity and expression of PKA in different brain regions (cerebellum, frontal, temporal, occipital and parietal cortex) from autism and control subjects.

BODY

Brain region–specific deficit in mitochondrial electron transport chain complexes in children with autism. There is lack of knowledge of the causative factors and secondary abnormalities in biochemical pathways in autism. Emerging evidence suggests increased prevalence of mitochondrial dysfunction in autism [20]. Since mitochondria play important roles in the generation of free radicals and ATP formation, we studied the levels of mitochondrial ETC complexes, i.e., complexes I, II, III, IV, and V, in brain tissue samples from the cerebellum and the frontal, parietal, occipital, and temporal cortices of autism and age-matched control subjects [8] (Appendix 1).

The postmortem frozen brain samples from the cerebellum and frontal, temporal, parietal and occipital cortex from autistic subjects with age range of 4 to 39 yrs from subjects with autism and age-matched control subjects were obtained from the National Institute of Child Health and Human Development (NICHD) Brain and Tissue Bank for Developmental Disorders at the University of Maryland.

The levels of mitochondrial ETC complexes were evaluated in homogenates from different brain regions of autistic and control subjects. The age (mean ± S.E.) for subjects with autism was 14.1 ± 4.5 y, and for control subjects, 14 ± 4.7 y. The samples were divided into two groups according to their ages: Group A (children, 4–10 years) and Group B (adults, 14–39 years). The tissue samples were homogenized (10% w/v) in cold buffer containing 50 mM Tris-HCl (pH 7.4), 8.5% sucrose, 2 mM EDTA, 10 mM β-mercaptoethanol, and protease inhibitor cocktail at 4 ^{0}C. The protein concentration was assayed by bicinchoninic acid (BCA) protein assay kit. The proteins in the brain homogenates of subjects with autism and control subjects

were detected by western blotting using primary mouse monoclonal OXPHOS antibody against mitochondrial ETC complexes I–V, and horseradish peroxidase–conjugated secondary antibody. The immunoreactive proteins were visualized using the ECL substrate. β-actin was used as the the loading control. The relative densities of the mitochondrial complexes vs. β-actin in autism and control groups were compared by unpaired student's t-test.

The levels of different ETC complexes were measured in the cerebellum (Fig. 1) and the frontal (Fig. 2), temporal (Fig. 3), parietal (Fig. 4), and occipital cortices (Fig. 5) of autism (A1-A8) and age-matched controls (C1-C8) by Western blotting. The relative density of the bands of different mitochondrial complexes vs. β-actin (loading control) is plotted as a histogram. Analysis of the data revealed lower levels of the ETC complexes in the cerebellum and the frontal and temporal cortices in the children with autism of ages 4-10 years (Group A) than in age-matched controls, but not in autistic group of 14-39 years of age (Group B). None of the ETC complexes showed any difference in parietal and occipital cortices between autistic and control subjects in any age group, suggesting that there are brain region–specific changes in mitochondrial ETC complexes in children with autism.

In Group A, we observed significantly lower levels of complexes III and V in the cerebellum, of complex I in the frontal cortex, and of complexes II, III, and V in the temporal cortex of children with autism as compared to age-matched control subjects (Figs. 1-3). None of the five ETC complexes was affected in the parietal and occipital cortices in subjects with autism (Fig. 4, 5). In the cerebellum and temporal cortex, no overlap was observed in the levels of these ETC complexes between autism and control subjects (Fig. 1, 3). In the frontal cortex of Group A, a lower level of ETC complexes was observed in a subset of autism cases, i.e., 60% for complexes I, II, and V, and 40% for complexes III and IV (Fig. 2). A striking observation was that the levels of ETC complexes were similar in adult autism and control subjects (Group B) (Figs. 1-5). These results suggest that the expression of mitochondrial ETC complexes is decreased in the cerebellum and the frontal and temporal regions of the brain in children with autism, which may lead to abnormal energy metabolism and oxidative stress [8] (Appendix 1). The deficits observed in the levels of ETC complexes in children with autism may readjust to normal levels by adulthood.

Brain Region–Specific Decrease in the Activity and Expression of Protein Kinase A in the Frontal Cortex of Regressive Autism. In regressive autism, affected children first show signs of normal social and language development but eventually lose these skills and develop autistic behavior. The underlying mechanism for regression in autism is not known. Protein kinases are essential in G-protein–coupled, receptor-mediated signal transduction and are involved in neuronal functions, gene expression, memory, and cell differentiation. We studied the activity and expression of protein kinase A (PKA), a cyclic AMP–dependent protein kinase, in postmortem brain tissue samples from the frontal, temporal, parietal, and occipital cortices, and the cerebellum of individuals with regressive autism; autistic subjects without a clinical history of regression; and age-matched developmentally normal control subjects [21](Appendix 2).

The activity of PKA (Fig. 6) and the expression of PKA (C-α) (Fig. 7), a catalytic subunit of PKA, were significantly decreased in the frontal cortex of individuals with regressive autism compared to control subjects and individuals with non-regressive autism. Such changes were not observed in the cerebellum, or the cortices from the temporal, parietal, and occipital regions of the brain in subjects with regressive autism (Fig. 6). In addition, there was no significant difference in PKA activity or expression of PKA (C-α) between non-regressive autism and

control groups (Fig. 6). These results suggest that regression in autism may be associated, in part, with decreased PKA-mediated phosphorylation of proteins and abnormalities in cellular signaling.

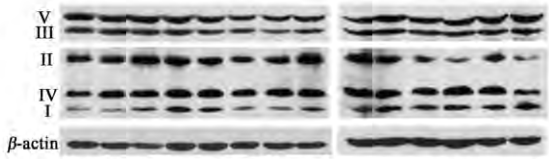

Fig. 1A (Group A) Fig. 1B (Group B)

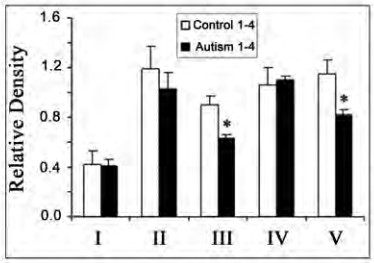

Fig. 1C (Group A)

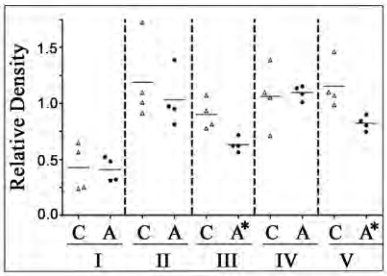

Fig. 1D (Group A)

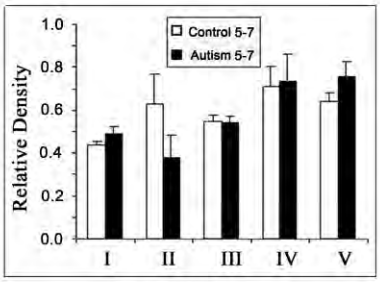

Fig. 1E (Group B)

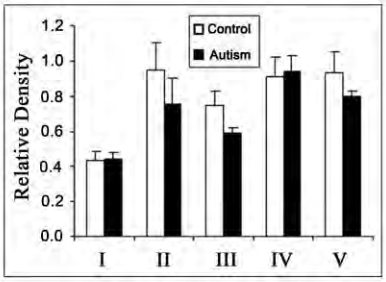

Fig. 1F (Group A+B)

Fig. 1. Electron transport chain complexes in the cerebellum from subjects with autism and age-matched control subjects in Group A (age: 4–10 years) and Group B (age: 14–39 years). In Group A, decreased levels of ETC complexes III and V were observed in the cerebellum of children with autism as compared to age-matched control subjects. In Group B, the levels of ETC complexes were similar in adult autistic and control subjects.

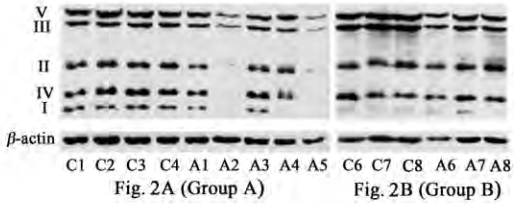

Fig. 2A (Group A) Fig. 2B (Group B)

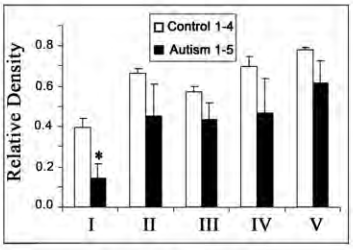

Fig. 2C (Group A)

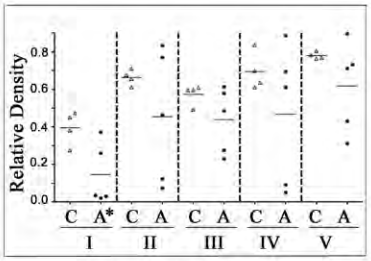

Fig. 2D (Group A)

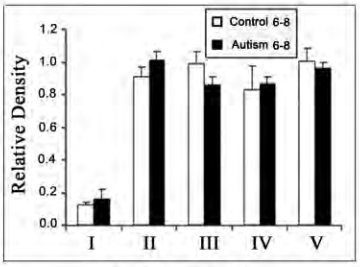

Fig. 2E (Group B)

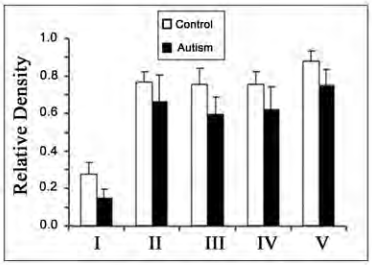

Fig. 2F (Group A+B)

Fig. 2. Electron transport chain complexes in the frontal cortex from subjects with autism (A1-A8) and age-matched control subjects (C1-C8) in Group A (age 4–10 years) and Group B (age 14–39 years). In Group A, a significant decrease in levels was observed for only complex I in autistic children than in control subjects; however, a general trend towards decreases in levels of the other complexes, i.e., II–V was also observed. In Group B, no change was observed in the levels of ETC complexes in autism.

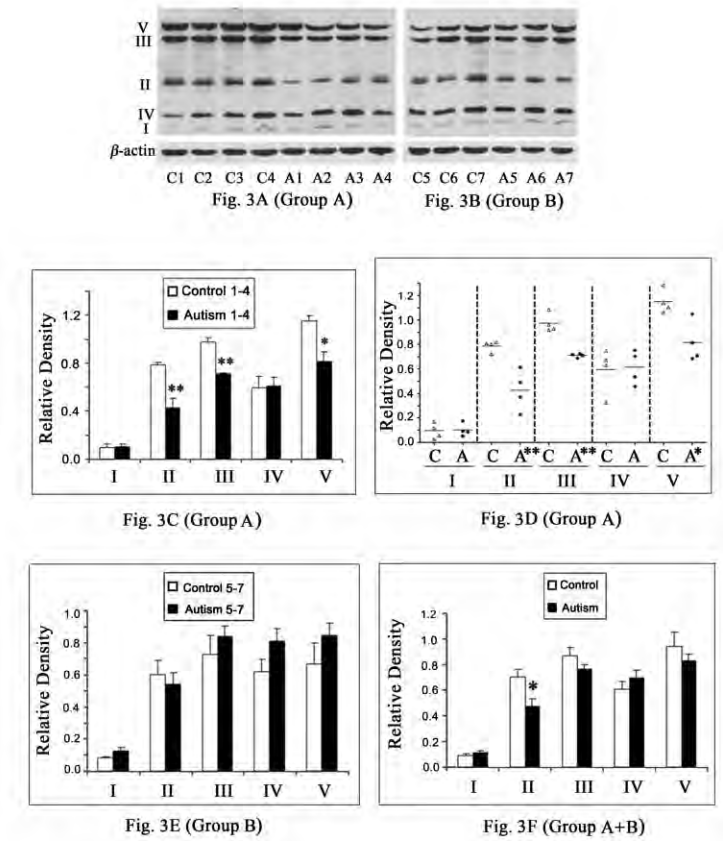

Fig. 3. Electron transport chain complexes in the temporal cortex from subjects with autism (A1-A7) and age-matched control subjects (C1-C7) in Group A (age 4–10 years) and Group B (age 14–39 years). ETC complexes II, III and V were significantly decreased in the temporal cortex of children with autism (Group A) but not in adult autistic subjects (Group B) in comparison with age-matched control subjects.

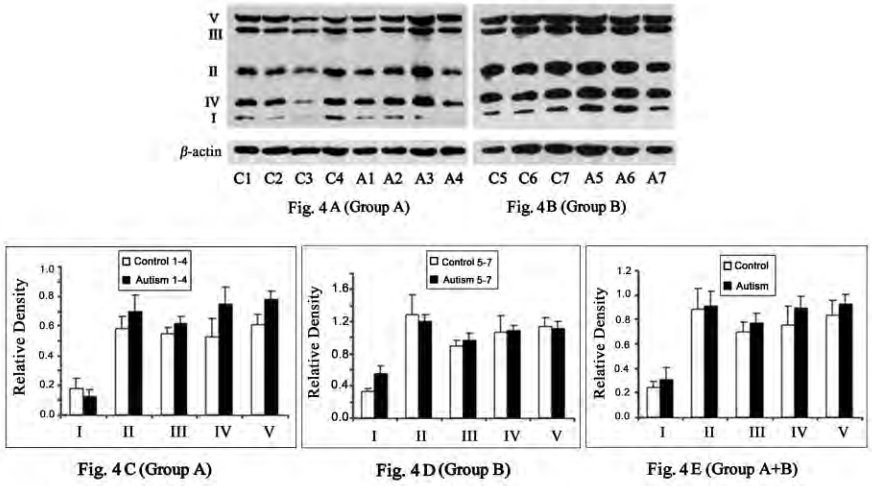

Fig. 4. Electron transport chain complexes in the parietal cortex from subjects with autism (A1-A7) and age-matched control subjects (C1-C7) in Group A (age 4–10 years) and Group B (age 14–39 years). No effect was observed on ETC complexes in the parietal cortex from subjects with autism (children and adults) as compared to age-matched control subjects.

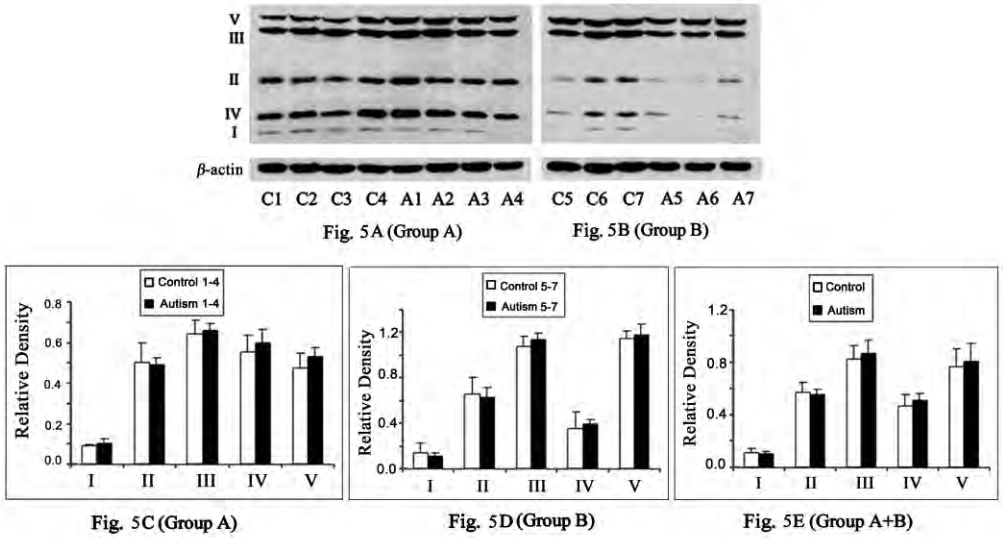

Fig. 5. Electron transport chain complexes in the occipital cortex from subjects with autism (A1-A7) and age-matched control subjects (C1-C7) in Group A (age 4–10 years) and Group B (age 14–39 years). No effect was observed on ETC complexes in the occipital cortex from subjects with autism (children and adults) as compared to age-matched control subjects.

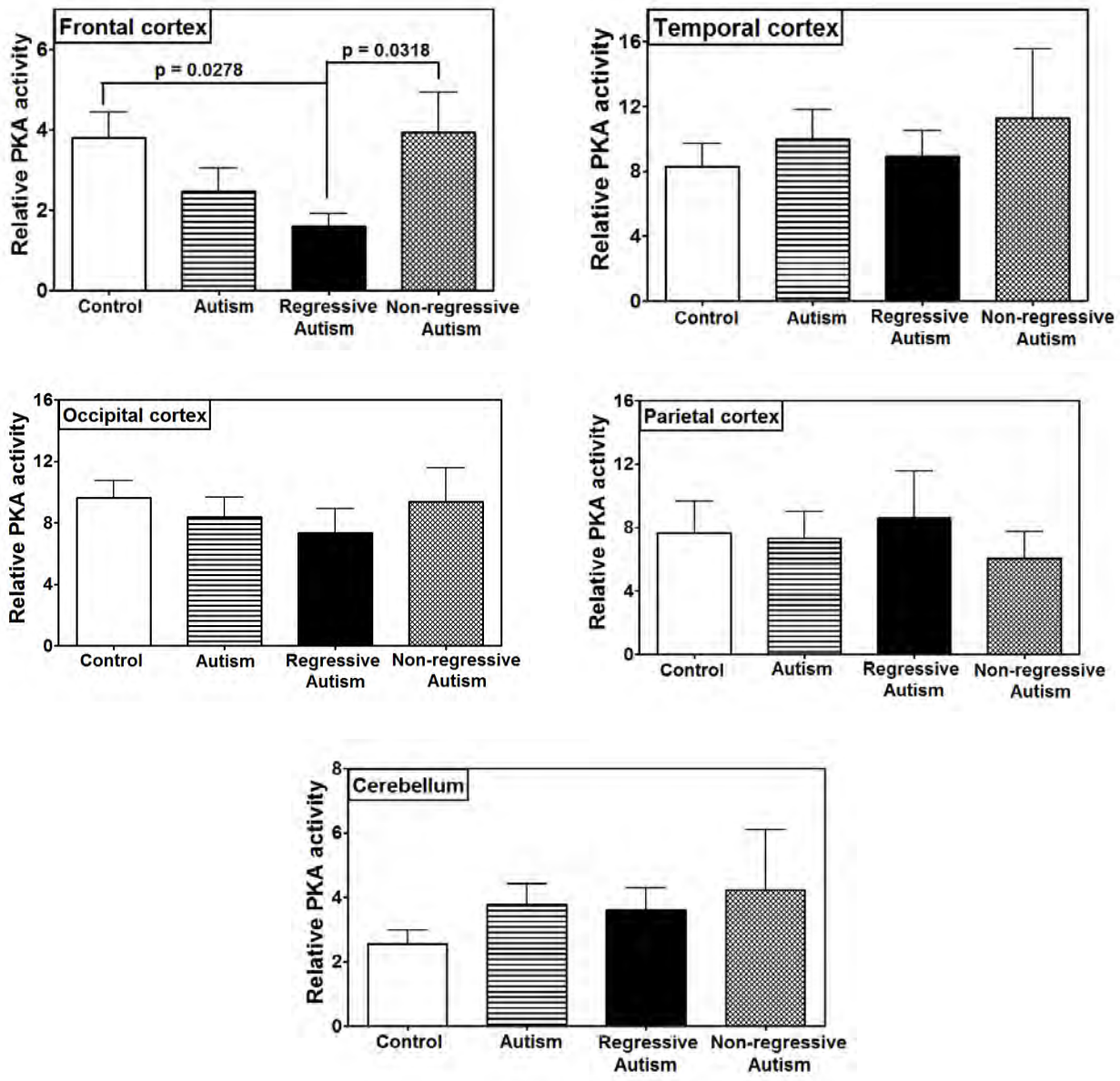

Figure 6. PKA activity in different brain regions from regressive autism, non-regressive autism, and age-matched control subjects. The autism group comprises combined regressive and non-regressive autism sub-groups. Data represent mean ± S.E.

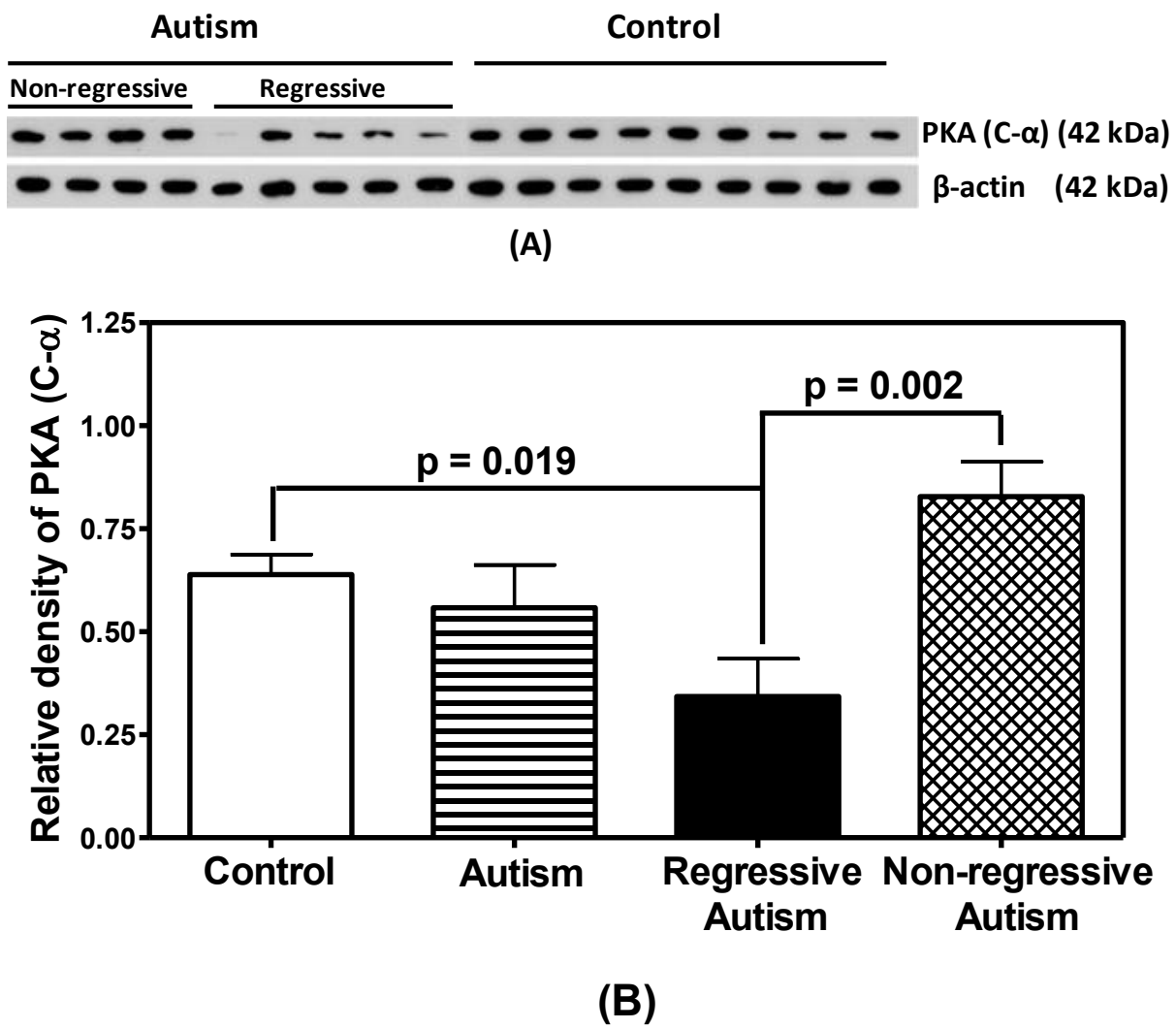

Figure 7. Relative protein levels of PKA (C-α) in the frontal cortex of regressive autism, non-regressive autism, and age-matched control subjects. Western blot analyses of C-α subunit of PKA in the frontal cortex of individuals with regressive and non-regressive autism, and age-matched control subjects are represented in Fig. 7A. The relative density of PKA (C-α) normalized with the density of β-actin (loading control) is shown in Fig. 7B. Data represent mean ± S.E.

KEY RESEARCH ACCOMPLISHMENTS

1. There is a brain region–specific decrease in the levels of mitochondrial electron transport chain complexes in the cerebellum and in the frontal and temporal cortices but not in the parietal and occipital cortices of subjects with autism [8]. These mitochondrial abnormalities are observed only in young children with autism but not in adults with autism. It is proposed that the abnormalities in the mitochondrial ETC complex levels resulting in disruption of mitochondrial function may be one of the factors in the etiology of autism. This will lead to increased free radical generation, oxidative stress and abnormal energy metabolism in autism.

2. There is increased oxidative damage [8-11] coupled with reduced antioxidant capacity [14] in the cerebellum, frontal cortex and temporal cortex but not in occipital and parietal cortex in autism.

3. Individuals with regressive autism have decreased PKA activity in the frontal cortex of the brain. This decreased PKA activity in autistic regression may be attributed to the decreased protein contents of PKA because the protein content of PKA (C-α subunit) was also decreased in the frontal cortex of individuals with regressive autism. Such changes were not observed in other brain regions of individuals with regressive autism, or in the frontal cortex and other brain regions of individuals with non-regressive autism. These results suggest that alterations in PKA activity and PKA expression are specific to the frontal lobe in regressive autism [21].

Our results suggest mitochondrial dysfunction, increased oxidative damage coupled with reduced antioxidant status in the specific regions of brain i.e., cerebellum, frontal and temporal cortex of autistic individuals compared with brain samples from age-matched control subjects. Frontal cortex may be the region of the brain involved in regressive autism, where abnormalities such as decreased activity and expression of PKA can affect the signal transduction.

REPORTABLE OUTCOMES

Abstracts

1. Chauhan A, Audhya T, Chauhan V. Increased DNA oxidation in the cerebellum, frontal and temporal cortex of brain in autism. Transactions of the American Society for Neurochemistry, 2011; p. 81.

2. Chauhan A, Audhya T, Chauhan V. Glutathione redox imbalance and increased DNA oxidation in specific brain regions in autism. International Meeting for Autism Research (Abstract), May 2011.

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5. Ji, L., Chauhan, V., Flory, M.J. and Chauhan, A. Brain region–specific decrease in the activity and expression of protein kinase A in the frontal cortex of regressive autism. PLoS ONE (in press)
6. Chauhan, A., Gu, F. and Chauhan, V. Mitochondrial respiratory chain defects in autism and other neurodevelopmental disorders. Special Issue: Mitochondrial dysfunction associated with neurodevelopmental disorders. J. Pediatric Biochem. (Invited review; submitted)

News Release of above publication #3

1. **News release of our publication in J. Neurochemistry (**Chauhan et al. Brain region–specific deficit in mitochondrial electron transport chain complexes in children with autism). **by Simons Foundation Autism Research Initiative (March 17, 2011)**
   https://sfari.org/news-and-commentary/open-article/-/asset_publisher/6Tog/content/mitochondrial-function-disrupted-in-children-with-autism?redirect=%2Fnews-and-commentary%2Fall

2. **Our recent article in J. Neurochemistry (**Chauhan et al. Brain region–specific deficit in mitochondrial electron transport chain complexes in children with autism) was featured as key scientific article by **Global Medical Discovery**

   http://globalmedicaldiscovery.com/key-scientific-articles/brain-region-specific-deficit-in-mitochondrial-electron-transport-chain-complexes-in-children-with-autism/.

CONCLUSIONS

Brain is a heterogeneous organ where specific functions are attributed to specific regions. Our results suggest that autism is associated with mitochondrial dysfunction and increased oxidative stress in the brain, which differentially affects selective regions of the brain, i.e. cerebellum, frontal cortex and temporal cortex in autism. Our results also suggest lower activity and expression of PKA in the frontal lobe of regressive autism, which will lead to abnormal cellular signaling. Increased oxidative damage may also lead to inflammation because oxidative stress serves as a major upstream component in the signaling cascade involved in activation of redox-sensitive transcription factors and pro-inflammatory gene expression resulting in an inflammatory response.

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APPENDICES

1. Chauhan, A., Gu, F., Essa,M.M., Wegiel,J., Kaur, K., Brown, W. T. and Chauhan, V. Brain region–specific deficit in mitochondrial electron transport chain complexes in children with autism. J. Neurochem. 117: 209-220 (2011).

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Brain region-specific deficit in mitochondrial electron transport chain complexes in children with autism

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Abstract

Mitochondria play important roles in generation of free radicals, ATP formation, and in apoptosis. We studied the levels of mitochondrial electron transport chain (ETC) complexes, that is, complexes I, II, III, IV, and V, in brain tissue samples from the cerebellum and the frontal, parietal, occipital, and temporal cortices of subjects with autism and age-matched control subjects. The subjects were divided into two groups according to their ages: Group A (children, ages 4–10 years) and Group B (adults, ages 14–39 years). In Group A, we observed significantly lower levels of complexes III and V in the cerebellum ($p < 0.05$), of complex I in the frontal cortex ($p < 0.05$), and of complexes II ($p < 0.01$), III ($p < 0.01$), and V ($p < 0.05$) in the temporal cortex of children with autism as compared to age-matched control subjects, while none of the five ETC complexes was affected in the parietal and occipital cortices in subjects with autism. In the cerebellum and temporal cortex, no overlap was observed in the levels of these ETC complexes between subjects with autism and control subjects. In the frontal cortex of Group A, a lower level of ETC complexes was observed in a subset of autism cases, that is, 60% (3/5) for complexes I, II, and V, and 40% (2/5) for complexes III and IV. A striking observation was that the levels of ETC complexes were similar in adult subjects with autism and control subjects (Group B). A significant increase in the levels of lipid hydroperoxides, an oxidative stress marker, was also observed in the cerebellum and temporal cortex in the children with autism. These results suggest that the expression of ETC complexes is decreased in the cerebellum and the frontal and temporal regions of the brain in children with autism, which may lead to abnormal energy metabolism and oxidative stress. The deficits observed in the levels of ETC complexes in children with autism may readjust to normal levels by adulthood.

Keywords: autism, electron transport chain complexes, energy, mitochondria, oxidative stress.

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Autism is a complex pervasive developmental disorder that is characterized by impaired language, communication, and social skills, as well as by repetitive and stereotypic patterns of behavior, all occurring by the age of 3 years (Lord *et al.* 2000). It is a heterogeneous disorder, belonging to a group of neurodevelopmental disorders, known as the autism spectrum disorders (ASDs) that include Asperger syndrome and pervasive developmental disorder-not otherwise specified. According to a recent report from the Centers for Disease Control and Prevention, the prevalence of autism by the age of 8 years is 1 in 110 children (Rice 2009). The onset of autism is gradual in many children. However, functional regression has been reported in early childhood in some autism cases (Goldberg *et al.* 2003; Lord *et al.* 2004; Ozonoff *et al.* 2005; Hansen *et al.* 2008). Accumulating evidence supports a prenatal onset for developmental abnormalities leading to autism (Kolevzon *et al.* 2007; Kinney *et al.* 2008). Postmortem assessments of the brains of individuals with autism have unveiled early neurodevelopmental alterations, including reduced programed cell death and/or increased cell proliferation, altered cell migration, abnormal cell differentiation with reduced neuronal size, and altered synaptogenesis (Bauman and Kemper 2005; Wegiel *et al.* 2009, 2010).

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Abbreviations used: ASDs, autism spectrum disorders; ETC, electron transport chain; LOOH, lipid hydroperoxide; ROS, reactive oxygen species.

Mitochondria are central to many cellular functions, including the generation of energy in the form of ATP and the maintenance of intracellular calcium homeostasis. They are the primary source of free radicals, that is, reactive oxygen species (ROS) and trigger apoptosis (Cadenas and Davies 2000; Lenaz 2001; Szewczyk and Wojtczak 2002; Polster and Fiskum 2004). Neurons in particular rely on the mitochondria because of neurons' high levels of metabolism and subsequent need for energy. Mitochondria are localized in synapses, and alterations of the number, morphology, or function of synaptic mitochondria can be detrimental to synaptic transmission (Polster and Fiskum 2004). Extensive evidence suggests that mitochondrial dysfunction, oxidative stress, and reduced neurotransmission occur in the early stages of several major neurodegenerative diseases, such as Alzheimer's disease (Reddy 2008; Reddy and Beal 2008; Aliev *et al.* 2009; Wang *et al.* 2009), Parkinson's disease (Schapira *et al.* 1990; Navarro *et al.* 2009), Huntington disease (Gu *et al.* 1996), and amyotrophic lateral sclerosis (Wiedemann *et al.* 2002). Mitochondrial decay has also been suggested to be major contributor to aging (Ames 2004; Reddy 2008). In addition, mitochondrial dysfunction in the brain of some individuals with schizophrenia has been reported (Bubber *et al.* 2004). However, brain mitochondria have not yet been studied in autism, although altered energy metabolism as evidenced by alterations in peripheral markers, such as increased plasma lactate levels has been suggested in autism (Filipek *et al.* 2004; Correia *et al.* 2006).

Mitochondria are responsible for most of the energy production through oxidative phosphorylation, a process requiring the action of various respiratory enzyme complexes, the mitochondrial electron transport chain (ETC) located in the inner mitochondrial membrane (Szewczyk and Wojtczak 2002; Boekema and Braun 2007). Mitochondria produce ATP by generating a protons gradient (membrane potential) with the help of five ETC complexes, that is, complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome *bc*1 complex), complex IV (cytochrome *c* oxidase), and ATP synthase, also known as complex V, where the electron transport couples with translocation of protons from the mitochondrial matrix to the intermembrane space. The generated proton gradient is used by ATP synthase to catalyze the formation of ATP by the phosphorylation of ADP (Scholes and Hinkle 1984; Bertram *et al.* 2006). The number of mitochondria per cell is roughly related to the energy demands of the cell. The brain has a high demand for energy, and neurons contain a large number of mitochondria. The ETC in mitochondria is also a prime mechanism for free radicals generation (Cadenas and Davies 2000; Lenaz 2001). The changes in the mitochondrial ETC have been suggested to be an important factor in the pathogenesis of several diseases, including neuropsychiatric (Rezin *et al.* 2009) and neurodegenerative disorders (Burchell *et al.* 2010; Moreira *et al.* 2010).

In this study, we compared the protein levels of various mitochondrial respiratory ETC complexes in different regions of the brain from subjects with autism and age-matched control subjects. Although children with autism showed a decrease in protein levels of ETC complexes in the cerebellum and the frontal and temporal cortices, no change was observed in the occipital and parietal cortices. Interestingly, when we analyzed the data as a function of age, children with autism (4–10 years of age) but not adults with autism (14–39 years of age) showed lower protein levels of brain ETC complexes, suggesting that developmental mitochondrial abnormalities resulting in mitochondrial dysfunction, oxidative stress, and abnormal energy metabolism may contribute to autistic phenotype.

Materials and methods

Materials

Samples of postmortem frozen brain regions, that is, the cerebellum, and cortices from the frontal, temporal, parietal, and occipital lobes (N = 7–8 for different brain regions) from subjects with autism and age-matched control subjects were obtained from the National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders at the University of Maryland. Donors with autism fit the diagnostic criteria of the Diagnostic and Statistical Manual-IV, as confirmed by the Autism Diagnostic Interview-Revised. All brain samples were stored at −70°C. This study was approved by the Institutional Review Board of the New York State Institute for Basic Research in Developmental Disabilities. The case history (diagnosis, age, postmortem interval, and cause of death) for the subjects with autism and control subjects is summarized in Table S1.

Preparation of brain homogenates

The tissue samples were homogenized (10% w/v) in cold buffer containing 50 mM Tris-HCl (pH 7.4), 8.5% sucrose, 2 mM EDTA, 10 mM β-mercaptoethanol, and protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA) in a Downs homogenizer with five strokes at 4°C. The protein concentration was assayed by bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL, USA).

Western blotting

The brain homogenates of subjects with autism and control subjects were mixed with loading buffer and boiled in a water bath for 5 min. Fifty micrograms of total protein of each sample was separated using a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane (0.45 μm; Bio-Rad Laboratories, Hercules, CA, USA) using 100 V for 40 min. The membrane was blocked with Tris-buffered saline containing 5% fat-free dried milk for 1 h at 22°C, and further incubated overnight at 4°C with mouse monoclonal OXPHOS antibody (dilution 1 : 1500; MitoSciences, Eugene, OR, USA) against mitochondrial ETC complexes I–V. The membrane was then washed with Tris-buffered saline-0.05% Tween 20 three times and incubated with horseradish peroxidase-conjugated secondary antibody (dilution 1 : 5000; Thermo Scientific) for 45 min at 22°C. The

membrane was washed again, and the immunoreactive proteins were visualized using the ECL substrate (Thermo Scientific). The levels of β-actin (the loading control) were determined by stripping and reprobing the membrane with anti-β-actin antibody (dilution 1 : 20 000; Abcam, Cambridge, MA, USA). While the two top bands for ETC complexes II and V were clearly visible, other complexes were relatively faint on the same blot. Therefore, to enhance visualization of the remaining ETC complexes, the membrane was re-exposed for a longer period of time.

The film was scanned and the bands were analyzed by using Image J software (NIH, Bethesda, MD, USA). The densities of different mitochondrial ETC complexes and β-actin were estimated. The relative densities of the mitochondrial complexes versus β-actin in autism and control groups were compared by unpaired Student's *t*-test.

Measurement of lipid hydroperoxide (LOOH)

The levels of LOOH were measured in the brain homogenates, as described by Patsoukis and Georgiou 2007. The photometric assay of LOOH measurement is based on the reaction of Fe^{3+} with LOOH, which converts Fe^{3+} to Fe^{2+}. The reaction of Fe^{2+} with the reagent dye xylenol orange results in the formation of chromogenic product, which is measured at 560 nm.

Results

The levels of different ETC complexes were measured in the cerebellum and the frontal, parietal, occipital, and temporal cortices of autism and age-matched controls by western blotting. The relative density of the bands of different mitochondrial complexes versus β-actin (loading control) is plotted as a histogram, and scattered plots show overall distribution of the data. Analysis of the data revealed lower levels of the ETC complexes in the cerebellum and the frontal and temporal cortices in the children with autism of ages 4–10 years than in age-matched controls, but not in autistic group of 14–39 years of age. None of the ETC complexes showed any difference in parietal and occipital cortices between subjects with autism and control subjects in any age group, suggesting that there are brain region-specific changes in mitochondrial ETC complexes in children with autism. Therefore, we divided the samples into two groups: Group A (children, 4–10 years) and Group B (adults, 14–39 years). The densitometric data of all ETC complexes normalized to β-actin are shown for all brain regions in Group A, Group B, and the entire group, that is, Group A + Group B. The scattered plot of samples is only shown when statistically significant changes in ETC complexes between autism and control groups were observed.

Lower levels of ETC complexes III and V in the cerebellum of children with autism

Western blot analysis of the levels of different ETC complexes in the cerebellum of subjects with autism and age-matched control subjects is shown in Fig. 1(a) (Group A, age: 4–10 years) and Fig. 1(b) (Group B, age: 14–39 years). The relative densities of different ETC complexes normalized to that of β-actin (loading control) are presented in Fig. 1(c) (Group A), Fig. 1(e) (Group B), and Fig. 1(f) (Groups A + B). In Group A, significantly lower levels were observed for complex III [mean ± SE = 0.629 ± 0.032 (autism), 0.899 ± 0.067 (control), $p < 0.05$)] and complex V [mean ± SE = 0.823 ± 0.032 (autism), 1.154 ± 0.105 (control), $p < 0.05$)] in subjects with autism as compared with age-matched controls (Fig. 1c). Scattered plot of the data in Group A showed that there was no overlap for complexes III and V between subjects with autism and control subjects (Fig. 1d). A trend toward lower levels of complex II was also observed in subjects with autism compared to control subjects, but it was not significant (Fig. 1c), while the levels of complexes I and IV were similar between subjects with autism and control subjects. In adults, that is, Group B, there was no change in the levels of ETC complexes in subjects with autism compared with those in age-matched controls. However, a decrease in complex II was observed in 66% of subjects with autism (mean ± SE = 0.38 ± 0.102) compared with control subjects (mean ± SE = 0.626 ± 0.139), but it was not significant. When the data were analyzed for Group A + Group B, lower levels of complexes II, III, and V were observed in subjects with autism, but it was not significant (Fig. 1f).

Lower levels of ETC complexes in the frontal cortex of children with autism

Western blot analysis of the levels of ETC complexes in the frontal cortex of subjects with autism and age-matched control subjects is shown in Fig. 2(a) for Group A, and Fig. 2(b) for Group B. Histogram analysis of the relative density of the data of ETC complexes is shown in Fig. 2(c) for Group A, Fig. 2(e) for Group B, and Fig. 2(f) for the entire Group A + B. When data in Group A were analyzed, a significant decrease in levels was observed for only complex I [(mean ± SE = 0.143 ± 0.073 (autism), 0.395 ± 0.044 (control), $p < 0.05$)]; however, a general trend toward decreases in levels of the other complexes, that is, II–V was also observed (Fig. 2c). It was interesting to observe from the scattered plot that 60% (3/5) of complexes I, II, and V, and 40% (2/5) of complexes III and IV in the autism group had levels below the cutoff lower range for the control group, suggesting that a subset of autism cases has decreased levels of all ETC complexes in the frontal cortex.

In Group B, no change was observed in the levels of ETC complexes (Fig. 1e), except that a non-significant decrease was observed for complex III, where 66% of subjects with autism had decreased levels. When both Groups A and B were analyzed together (Fig. 2f), a general trend toward decreases in the levels of all ETC complexes was observed in subjects with autism, but it was not significant.

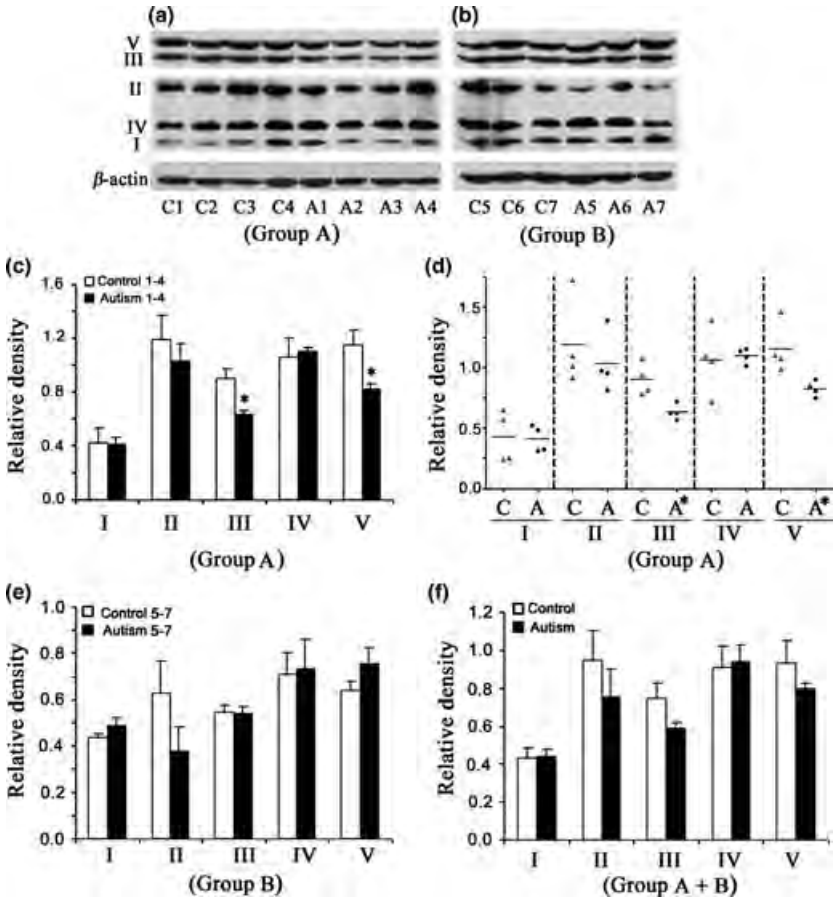

Fig. 1 Electron transport chain (ETC) complexes in the cerebellum from subjects with autism and age-matched control subjects in Group A (age: 4–10 years) and Group B (age: 14–39 years). The Group A samples were A1–A4 for subjects with autism, and C1–C4 for control subjects (a), whereas Group B samples were A5–A7 for subjects with autism, and C5–C7 for control subjects (b). Western blots are represented in (a) (Group A) and (b) (Group B). The relative densities of different ETC complexes normalized to β-actin are shown in (c) (Group A), (e)(Group B), and (f) (combined Groups A + B). Scattered plot of the data for Group A is shown in (d). *$p < 0.05$, unpaired *t*-test.

Lower levels of ETC complexes II, III and V in the temporal cortex of children with autism

Western blot analysis of the levels of ETC complexes in the temporal cortex of subjects with autism and age-matched control subjects is shown in Fig. 3(a) (Group A) and Fig. 3(b) (Group B). Data analysis in Group A showed that the levels of complexes II, III, and V were significantly lower in subjects with autism as compared with age-matched control subjects (Fig. 3c). The mean values ± SE were as follows: for complex II in autism, 0.425 ± 0.082, in control, 0.787 ± 0.022 ($p < 0.01$); complex III in autism, 0.710 ± 0.008, and in control, 0.972 ± 0.038 ($p < 0.01$); and complex V in autism, 0.813 ± 0.083, and in control, 1.147 ± 0.048 ($p < 0.05$). Scattered plot analysis showed that there was no overlap in the levels of these ETC complexes between subjects with autism and control subjects. In contrast, no significant change in any of the ETC complexes was observed in Group B (Fig. 5f). When both Groups A and B were combined, only complex II was significantly decreased ($p < 0.05$) in subjects with autism (mean ± SE = 0.474 ± 0.057) as compared to control subjects (mean ± SE = 0.706 ± 0.053) (Fig. 3f).

The levels of ETC complexes are not affected in parietal and occipital cortices of subjects with autism

Western blots of ETC complexes in the parietal cortex (Fig. 4a and b) and occipital cortex (Fig. 5a and b) and histograms of relative densities (parietal cortex, Fig. 4c–e; occipital cortex, Fig. 5c–e) showed that the levels of ETC complexes are not affected in Group A as well as in Group B of subjects with autism as compared to age-matched control subjects. These results suggest that there is a brain region-specific decrease in the levels of ETC complexes in the cerebellum and the frontal and temporal cortices but not in the parietal and occipital cortices of subjects with autism. Because the parietal and occipital cortices were not affected in subjects with autism in

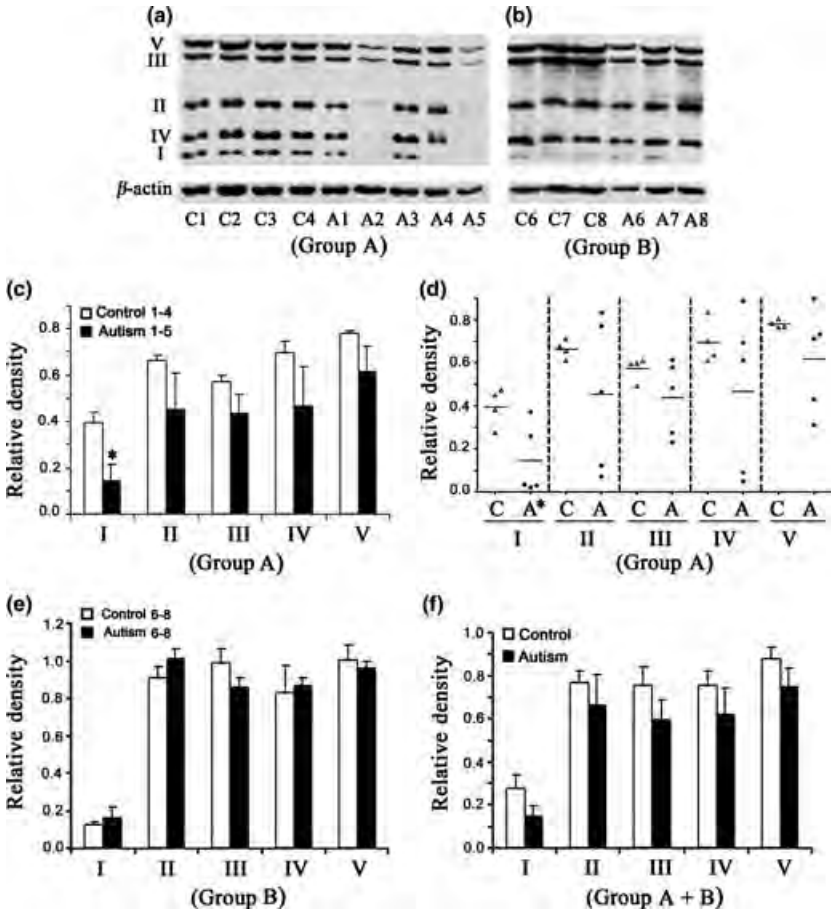

Fig. 2 Electron transport chain (ETC) complexes in the frontal cortex from subjects with autism and age-matched control subjects in Group A (age: 4–10 years) and Group B (age: 14–39 years). The Group A samples were A1–A5 for subjects with autism, and C1–C4 for control subjects (a), whereas Group B samples were A6–A8 for subjects with autism, and C5–C7 for control subjects (b). Western blots are represented in (a) (Group A) and (b) (Group B). The relative densities of different ETC complexes normalized to β-actin are shown in (c) (Group A), (e) (Group B), and (f) (combined Groups A + B). Scattered plot of the data for Group A is shown in (d). *$p < 0.05$, unpaired *t*-test.

comparison with control subjects, while the frontal and temporal cortices and the cerebellum from subjects with autism were affected, our results indirectly suggest that postmortem interval is not a contributing factor toward the observed brain mitochondrial abnormalities in autism.

Increased levels of LOOHs in specific brain regions in children with autism

To assess whether changes in mitochondrial ETC in the children with autism also results in increased free radical generation and oxidative stress, we measured the levels of LOOH, a product of fatty acid oxidation, in the frontal, temporal, occipital and parietal cortices, and cerebellum from children with autism and age-matched controls (Fig. 6). The levels of LOOH were significantly increased in the cerebellum and temporal cortex of subjects with autism as compared with age-matched control subjects in Group A. An increase in the levels of LOOH was also observed in the frontal cortex in autism group, but it was not statistically significant. No change in the levels of LOOH was observed in the parietal and occipital cortices between autism and control groups.

Discussion

Although the cause of autism remains elusive, it is considered a multifactorial disorder that is influenced by genetic, environmental, and immunological factors as well as increased vulnerability to oxidative stress (Chauhan and Chauhan 2006). In this study, we report two interesting observations: (i) brain region-specific changes occur in the levels of ETC complexes in the cerebellum and the frontal and temporal cortices but not in the parietal and occipital cortices in subjects with autism, and (ii) the changes above are observed only in young children with autism but not in adults with autism. We recently reported that the activities of Ca^{2+}-Mg^{2+}-ATPase and Na^{+}-K^{+}-ATPase were also affected

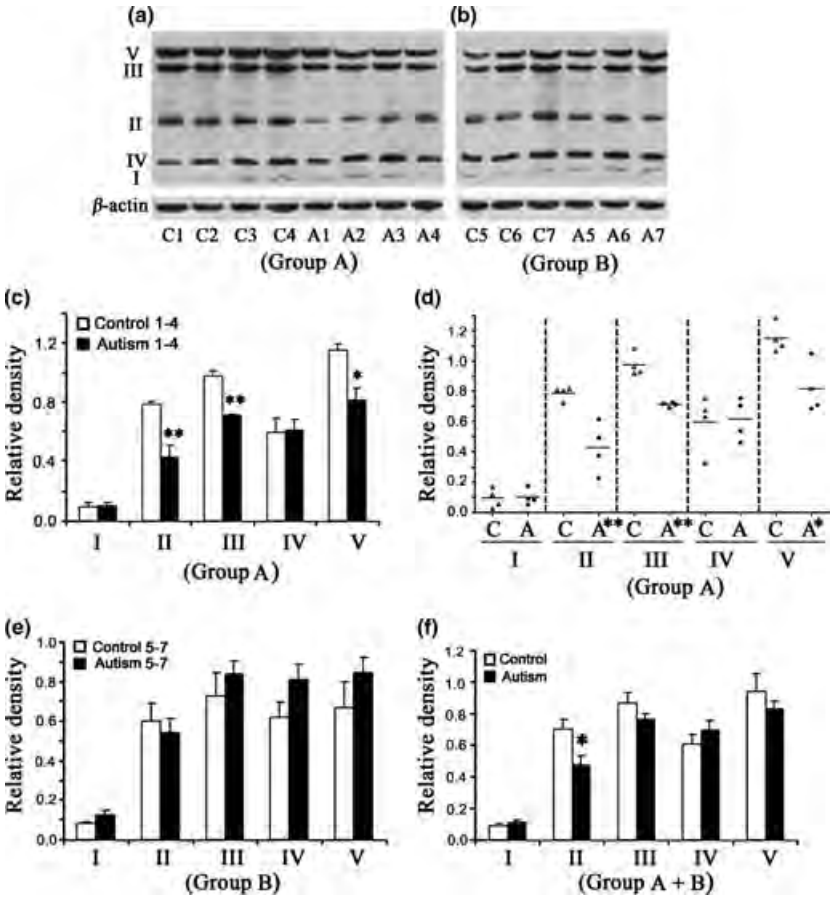

Fig. 3 Electron transport chain (ETC) complexes in the temporal cortex from subjects with autism and age-matched control subjects in Group A (age: 4–10 years) and Group B (age: 14–39 years). The Group A samples were A1–A4 for subjects with autism, and C1–C4 for control subjects (a), whereas Group B samples were A5–A7 for subjects with autism, and C5–C7 for control subjects (b). Western blots are represented in (a) (Group A) and (b) (Group B). The relative densities of different ETC complexes normalized to β-actin are shown in (c) (Group A), (e) (Group B), and (f) (combined Groups A + B). Scattered plot of the data for Group A is shown in (d). $^{*}p < 0.05$, $^{**}p < 0.01$, unpaired *t*-test.

in the cerebellum and frontal cortex, suggesting that the cerebellum and frontal cortex may have biochemical changes in autism (Ji *et al.* 2009). Mitochondria are vulnerable to a wide array of endogenous and exogenous factors, which appear to be linked by excessive production of free radicals. The free radicals are generated endogenously during oxidative metabolism and energy production by mitochondria (Cadenas and Davies 2000; Lenaz 2001). Mitochondria are not only the source of free radicals, but they are also the target of oxidative damage. In addition to producing more oxidants, damaged mitochondria are also vulnerable to oxidative stress. Increasing evidence from our and other groups suggests a role of oxidative stress in the development and clinical manifestation of autism (McGinnis 2004; Chauhan and Chauhan 2006). Levels of oxidative stress markers are increased in the blood (Chauhan *et al.* 2004; James *et al.* 2004; Zoroglu *et al.* 2004; Chauhan and Chauhan 2006), urine (Ming *et al.* 2005), and brains (Lopez-Hurtado and Prieto 2008; Evans *et al.* 2009; Muthaiyah *et al.* 2009; Sajdel-Sulkowska *et al.* 2009) of individuals with autism as compared with controls. In this study, increased levels of LOOH were also observed in the children with autism in same brain regions where mitochondrial ETC abnormalities were observed. As ETC in mitochondria is a prime source for ROS generation, these results also support the findings on mitochondrial dysfunction in children with autism.

Mitochondria play a central role in the energy-generating process through the transfer of electrons with the help of five ETC complexes and generation of a proton gradient in the inner membrane of the cell. Although the end product of the respiratory chain is water that is generated in a four-electron reduction of molecular oxygen by complex IV, a minor proportion of O_2 can be involved in the one-electron reduction processes generating ROS, in particular, superoxide anion radical ($\cdot O^-_2$), hydrogen peroxide (H_2O_2), and the extremely reactive hydroxyl radical ($OH\cdot$). Generation of ROS occurs mainly at complex III as a result of proton

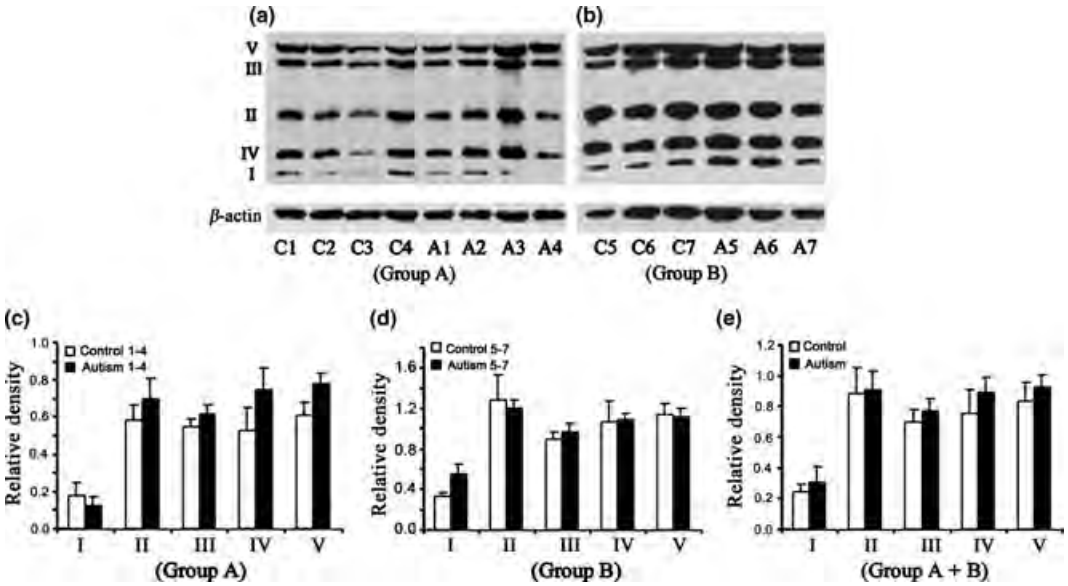

Fig. 4 Electron transport chain (ETC) complexes in the parietal cortex from subjects with autism and age-matched control subjects in Group A (age: 4–10 years) and Group B (age: 14–39 years). The Group A samples were A1–A4 for subjects with autism, and C1–C4 for control subjects (a), whereas Group B samples were A5–A7 for subjects with autism and C5–C7 for control subjects (b). Western blots are represented in (a) (Group A) and (b) (Group B). The relative densities of different ETC complexes normalized to β-actin are shown in (c) (Group A), (d) (Group B), and (e) (combined Groups A + B).

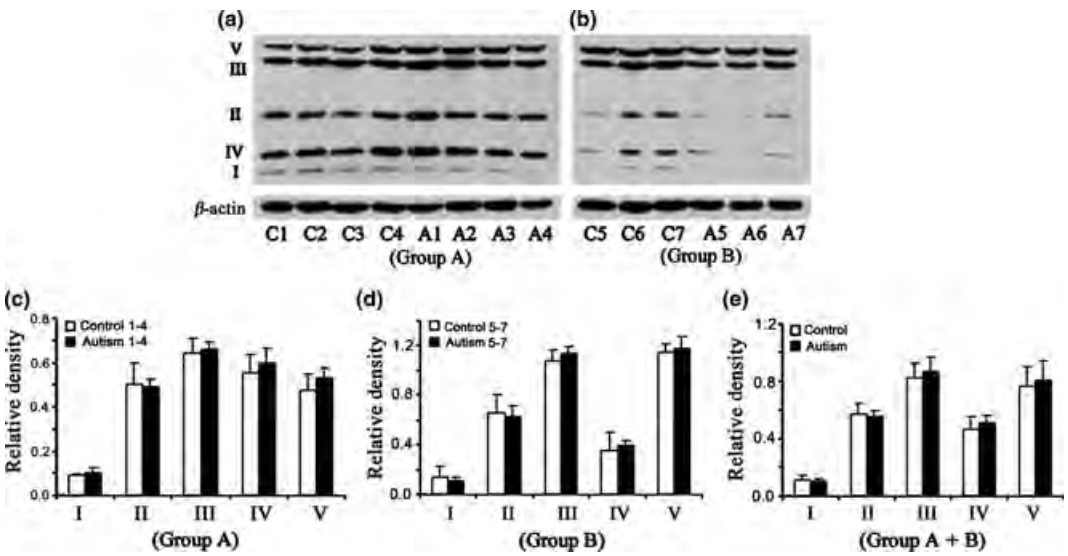

Fig. 5 Electron transport chain (ETC) complexes in the occipital cortex from subjects with autism and age-matched control subjects in Group A (age: 4–10 years) and Group B (age: 14–39 years). The Group A samples were A1–A4 for subjects with autism, and C1–C4 for control subjects (a), whereas group B samples were A5–A7 for subjects with autism and C5–C7 for control subjects (b). Western blots are represented in (a) (Group A) and (b) (Group B). The relative densities of different ETC complexes normalized to β-actin are shown in (c) (Group A), (d) (Group B), and (e) (combined Groups A + B).

cycling between ubiquinone, cytochromes *b* and *c*1, and iron-sulfur protein (Sugioka *et al.* 1988). Some contribution of complex I to this process has also been found. Consequently, abnormalities in the levels of ETC complexes may be responsible for the observed oxidative stress in autism. The brain is highly vulnerable to oxidative stress, as it represents only 2% of the total body weight, but it accounts for 20% of all oxygen consumption, reflecting its high rate of

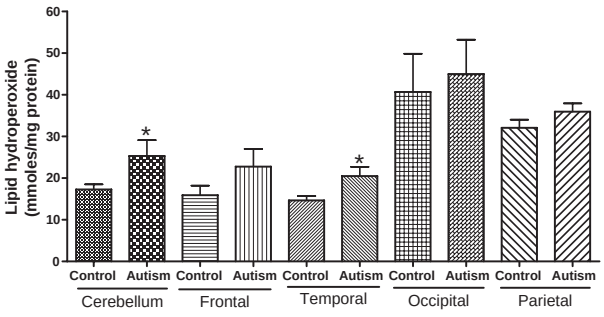

Fig. 6 Levels of lipid hydroperoxides in different regions of brain from subjects with autism and age-matched controls in Group A (age: 4–10 years). Lipid hydroperoxides were measured in the brain homogenates from frontal, temporal, occipital and parietal cortices, and cerebellum of subjects with autism and age-matched controls in Group A. The data represent mean ± SE. $^*p < 0.05$, unpaired *t*-test.

metabolic activity (Juurlink and Paterson 1998; Shulman *et al.* 2004). Mitochondria have a crucial role in the supply of energy to the brain. Damaged ETC complexes compromise ATP synthesis and accelerate the generation of free radicals. Therefore, the mitochondrial ETC defects observed in the brains of young individuals with autism may have important, detrimental consequences on the function and plasticity of neurons in autism.

Mitochondrial diseases have been linked to poor growth, loss of muscle coordination, muscle weakness, developmental delays, learning disabilities, mental retardation, gastrointestinal disorders, neurological problems, seizures, and dementia (Read and Calnan 2000; Xu *et al.* 2005; Aliev *et al.* 2009). Depending on how severe the mitochondrial disorder is, the illness can range in severity from mild to fatal. It should be noted that some of the symptoms of mitochondrial diseases, such as learning disabilities, mental retardation, seizures, neurological problems, and gastrointestinal disturbances, are also present in a subset of individuals with autism.

Although this study is the first to report on brain mitochondrial abnormalities in autism, a few case reports of mitochondrial disorder have been reported in individuals with autism on the basis of blood analysis and/or muscle biopsy. These case reports include a child with autism with documented complex IV deficiency (Laszlo *et al.* 1994); a boy with autism with complex IV defect and a mtDNA G8363A mutation (Graf *et al.* 2000); two children with autism with deficiencies in several respiratory chain enzymes, including complexes I–III and coenzyme Q (Tsao and Mendell 2007); and five individuals with autism and mtDNA mutations or a mtDNA deletion (Pons *et al.* 2004). Anatomical and neuroradiographical studies of the brains of individuals with autism have also suggested that a disturbance of energy metabolism may be present (Lombard 1998; Chugani *et al.* 1999). ^{31}P-Magnetic resonance spectroscopy showed increased membrane degradation and decreased synthesis of ATP in autism (Minshew *et al.* 1993). In addition, carnitine deficiency in plasma, accompanied by elevations in lactate, alanine, and ammonia levels in autism, findings suggestive of mild mitochondrial dysfunction was reported in autism (Filipek *et al.* 2004). Another study also showed a high frequency of increased plasma lactate levels and increased lactate/pyruvate ratio in individuals with autism (Correia *et al.* 2006). Although the mechanism of hyperlactacidemia remains unknown, these case reports support dysfunction of mitochondrial oxidative phosphorylation in autism.

A population-based study in Portugal examining medical conditions in 120 children with autism found a disproportionately high prevalence (7%) of mitochondrial diseases in individuals with autism (Oliveira *et al.* 2005). However, these children did not have any known mtDNA mutations and/or deletions associated with known mitochondrial disorders. This report suggests that a substantial percentage of subgroups of autism may have a mitochondrial disorder.

The risk of sudden death of individuals who have inverted duplication of chromosome 15q (idic 15) is approximately 1% per year (Cleary 2009). This abnormality occurs in 1–5% of individuals with autism (Gillberg 1998; Schroer *et al.* 1998). Children with autism with a chromosome 15q11-q13 inverted duplication have been found to have motor delay, lethargy, severe hypotonia, and modest lactic acidosis. It is of interest to note that two children with autism and idic 15 showed mitochondrial hyperproliferation and complex III defect (Filipek *et al.* 2003), and two autism cases associated with sudden infant death syndrome showed mild mitochondrial hyperproliferation and a possible complex II defect (Gargus and Imtiaz 2008). These studies suggest that candidate gene loci for autism within the critical region may affect pathways influencing mitochondrial function (Filipek *et al.* 2003).

In regressive autism, children first show signs of normal social and language development through the first year of life but lose these developmental skills at 15–24 months and develop autistic behavior (Ozonoff *et al.* 2005). The rate of regressive autism varies from 15% to 62% of cases (Goldberg *et al.* 2003; Lord *et al.* 2004; Hansen *et al.* 2008). A recent study examined a group of 25 individuals with autism who also had confirmed mitochondrial disorders (Weissman *et al.* 2008). They reported that 40% of this group demonstrated unusual pattern of regression (multiple episodes, loss of motor skills, and regression after the age of 3). In this cohort, the deficiency of ETC complexes I and III was observed in 64% and 20% of individuals with autism, respectively, and two had a rare mtDNA mutation. Another case report implicated mitochondrial dysfunction as a factor contributing to vaccine-related regression (Poling *et al.* 2006; Zecavati and Spence 2009). A recent report also suggests that fever in children with mitochondrial disease is a risk to autistic regression (Shoffner *et al.* 2010).

This study suggests that abnormalities in the mitochondrial ETC complex levels may be one of the factors in the etiology of autism. This will lead to oxidative stress and abnormal energy metabolism in autism. In our studies, deficiency of mitochondrial ETC complexes was observed in children with autism (ages 4–10 years) but not in adults with autism (14–39 years of age). Age also seems to play a critical role in determining brain growth in autism. Enlarged brain size (megaloencephaly), particularly in the temperoparietal region, is the most consistent observation in young children with autism (Goldberg *et al.* 1999). The initial accelerated brain growth in young children is followed by abnormal slowness and growth arrest that results in normalization of brain size in late childhood and in adults (Hardan *et al.* 2001; Aylward *et al.* 2002; Courchesne 2004; Herbert 2005). Head circumference measurements have also shown increased brain volume in young children, later returning to normal volume. Thus, very large differences between children with autism and normal children are evident at early ages, but differences are not seen in adult cases (Aylward *et al.* 2002; Courchesne 2004). In addition, age-related changes in cerebellar nuclei and inferior olives have also been reported in autism (Palmen *et al.* 2004). The pattern of age-related changes in the severity of autism symptoms also suggests that causative factors determine both developmental and age-associated modifications. While age-related increases in the severity of autism symptoms have been reported among individuals with idic 15 syndrome (Rineer *et al.* 1998), significant improvement of communication and social behaviors with increasing age has been reported in other autistic cohorts (Mesibov *et al.* 1989; Piven *et al.* 1996). Recent evidence suggests that 3–25% children with a previous diagnosis of ASD recover and show normal ranges of cognitive, adaptive, and social skills (Helt *et al.* 2008).

Neuropathological studies in autism suggest prenatal and postnatal developmental abnormalities in multiple regions of the brain, including the cerebellum, frontal and temporal cortices, cortical white matter, amygdala, and brainstem (particularly the olivary nuclei) (Palmen *et al.* 2004; Bauman and Kemper 2005; Pickett and London 2005; Schmitz and Rezaie 2008; Wegiel *et al.* 2009, 2010). There is substantial evidence from neuroimaging studies that dysfunctions in the cerebellum and possibly the temporal lobe and association cortex result in autism symptoms. Loss of Purkinje and granule cells has been reported throughout the cerebellar hemispheres in autism (Bauman and Kemper 1985, 2005; Kern 2003; Casanova 2007). Alterations in neuronal size, density, and dendritic branching in the cerebellum and limbic structures (hippocampus and amygdala) have also been reported in autism.

The prevalence rate of mitochondrial disease is about one in 5000–10 000 children (Skladal *et al.* 2003; Schaefer *et al.* 2004). In contrast, the prevalence rate for autism is 1 in 110 children (Rice 2009). As we observed a high percentage of changes in complexes I–III, and V in the cerebellum and frontal and temporal cortices of individuals with autism, it seems that autism is associated with mitochondrial dysfunction, although clinical symptoms of mitochondrial disease may be lacking. Therefore, mitochondrial dysfunctions rather than mitochondrial disorders may be more relevant in autism. The clinical diagnosis of mitochondrial disease is often made with biochemical analysis of lactate, pyruvate, and alanine in blood, urine, or cerebrospinal fluid. However, the analysis of biochemical metabolites to diagnose mitochondrial disease may not be sufficient, as these analyses seem to be frequently normal, even in some severe cases of the disease. The clinical symptoms of mitochondrial disease are increased when ASD has comorbidity, such as hypotonia and motor delay, fatigue, metabolic abnormalities, and epilepsy (Fillano *et al.* 2002). The genetics of autism is complex, with the involvement of multiple genes. However, no gene has been identified that follows the typical Mendelian laws of inheritance. ASDs may have mild changes in the levels of ETC complexes that may or may not be related to a gene mutation. The mild form of mitochondrial abnormalities observed in autism may also be linked to other abnormalities such as the excessive Ca^{2+} observed in the mitochondria in autism. Excessive levels of Ca^{2+} in the mitochondria can affect the mitochondrial metabolism and increase the oxidative stress in the brains of individuals with autism (Palmieri *et al.* 2010).

The mechanism by which mitochondrial dysfunction may occur and affect development of autism is not entirely clear. It is possible that in comparison with classical mitochondrial disease, mitochondrial dysfunction may show less severe symptoms and may not show the classical mitochondrial pathology on muscle biopsy (Lombard 1998). Further research with larger sample sizes is needed to determine the association between mitochondrial dysfunction and severity, clinical phenotypes, regression, and/or idic 15 in autism.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1. Case history of autism and control brain samples.

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Brain Region–Specific Decrease in the Activity and Expression of Protein Kinase A in the Frontal Cortex of Regressive Autism

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Abstract

Autism is a severe neurodevelopmental disorder that is characterized by impaired language, communication, and social skills. In regressive autism, affected children first show signs of normal social and language development but eventually lose these skills and develop autistic behavior. Protein kinases are essential in G-protein-coupled, receptor-mediated signal transduction and are involved in neuronal functions, gene expression, memory, and cell differentiation. We studied the activity and expression of protein kinase A (PKA), a cyclic AMP–dependent protein kinase, in postmortem brain tissue samples from the frontal, temporal, parietal, and occipital cortices, and the cerebellum of individuals with regressive autism; autistic subjects without a clinical history of regression; and age-matched developmentally normal control subjects. The activity of PKA and the expression of PKA (C-α), a catalytic subunit of PKA, were significantly decreased in the frontal cortex of individuals with regressive autism compared to control subjects and individuals with non-regressive autism. Such changes were not observed in the cerebellum, or the cortices from the temporal, parietal, and occipital regions of the brain in subjects with regressive autism. In addition, there was no significant difference in PKA activity or expression of PKA (C-α) between non-regressive autism and control groups. These results suggest that regression in autism may be associated, in part, with decreased PKA-mediated phosphorylation of proteins and abnormalities in cellular signaling.

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Introduction

Autism spectrum disorders (ASDs) are neurodevelopmental disorders characterized by impairment in social interactions and verbal/non-verbal communication skills, and restricted, repetitive and stereotyped patterns of behavior [1]. According to a recent report from the Centers for Disease Control and Prevention, the prevalence of ASDs is 1 in 110 for children 8 years of age [2]. The symptoms of ASDs are typically present before the age of 3 years, and are often accompanied by abnormalities in cognitive functioning, learning, attention, and sensory processing. While the causes of ASDs remain elusive, ASDs are considered to be heterogeneous and multifactorial disorders that are influenced by both genetic and environmental factors. The onset of autism is gradual in many children. However, in regressive autism, children first show signs of normal social and language development but lose these developmental skills at 15–24 months and develop autistic behavior [3]. The reported incidence of regressive autism varies in different studies from 15% to 62% of cases [4–7]. In a few cases, regression may significantly affect language, with lesser impact in other domains such as social interaction or imaginative play [4,8]. On the other hand, some children may regress especially in social functions and not in language [9].

Protein kinases are known to play important roles in cellular signaling pathways and are involved in brain development [10–13]. Protein kinase A (PKA) is a cyclic adenosine monophosphate (cAMP)–dependent protein kinase that is involved in cognitive functions and memory formation [14–18]. PKA consists of regulatory (R) and catalytic (C) subunits. Three genes encode for catalytic units (Cα, Cβ, and Cγ), and four other genes encode for regulatory units (RIα, RIβ, RIIα, and RIIβ) of PKA. PKA remains catalytically inactive when the levels of cAMP are low. The concentration of cAMP rises upon activation of adenylate cyclase by G protein-coupled receptors, and/or inhibition of cyclic nucleotide phosphodiesterase (PDE) enzyme. Under these conditions, cAMP binds to two binding sites on the regulatory subunits of PKA, which results in the release of the catalytic subunits. These free catalytic units of PKA can then phosphorylate proteins by transferring a phosphate group from ATP. Several studies have implicated the role of PKA in neuropsychiatric disorders such as schizophrenia, bipolar affective disorder, obsessive compulsive disorder, and panic disorders [19–22]. To date, no studies of PKA have been done in autism.

The intracellular levels of cAMP are controlled by PDE, which degrades the phosphodiester bond in cAMP. PDE regulates the localization, duration, and amplitude of cAMP signaling within subcellular domains. Multiple forms of PDEs have been identified

on the basis of substrate specificity. PDE4, 7, and 8 act on cAMP; PDE5, 6, and 9 act on cyclic guanosine monophosphate (cGMP); whereas PDE1, 2, 3, 10, and 11 act on both cAMP and cGMP. Recent evidence has suggested altered levels of PDE4 in the brains of individuals with autism [23].

Because the levels of PDE4 are altered in autism, and PKA is involved in neuropsychiatric disorders, it was of interest to compare the activity and protein levels of PKA in different brain regions in autism (regressive and non-regressive) and age-matched control subjects. Our study suggests that PKA activity and expression are decreased in the frontal cortex of individuals with regressive autism as compared with control subjects. Such changes were not observed in individuals with non-regressive autism.

Materials and Methods

Autism and Control Subjects

Samples of postmortem frozen brain regions, i.e., the cerebellum, and the cortices from the frontal, temporal, parietal, and occipital lobes from autistic (N = 7–10 for different brain regions) and age-matched, typically developed, control subjects (N = 9–10) were obtained from the National Institute of Child Health and Human Development (NICHD) Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. The age (mean ± S.E.) for autistic subjects was 12.6±3.2 years, and for control subjects, 12.4±3.3 years. All brain samples were stored at −70°C.

The case history and clinical characteristics for the autism and control subjects are summarized in Table 1. Donors with autism had met the diagnostic criteria of the Diagnostic and Statistical Manual-IV for autism. The Autism Diagnostic Interview-Revised (ADI-R) test was performed for the donors UMB #s 4671, 4849, 1174, 797, 1182, 4899, and 1638 (Table 2). Each donor's impairments in social interaction, qualitative abnormalities in communication, and restricted, repetitive and stereotyped patterns of behavior are consistent with the diagnosis of autism, according to the results of the ADI-R diagnostic algorithm. All donors with autism exceeded the cut-off score in these parameters. The diagnosis of autism was assigned to donor UMB # 1349 after extensive evaluation of behavioral tests, including the Autism Diagnostic Observation Schedule (ADOS), Vineland Adaptive Behavioral Scale (VABS), and Bayley Scales for Infant Development-II (BSID-II). In addition to the ADI-R, UMB # 4849 was also evaluated by the BSID-II and Childhood Autism Rating Scale (CARS), which indicated moderate to severe autism, and autism in UMB # 4671 was also verified by the VABS and BSID-II. Table 3 shows scores for the VABS test, which assesses adaptive behavior in four domains: communication, daily living skills, socialization, and motor skills.

In this study, the subjects with autism were divided into two subgroups: regressive autism and non-regressive autism, depending on the pattern of onset of symptoms for autism. Regressive autism is a type of autism in which early development is normal, followed by a loss of previously acquired skills. Language is the most common area that regresses; this regression can be accompanied by more global regression involving loss of social skills and social interest. On the other hand, in non-regressive autism, the child never gains normal language and social skills, and initial symptoms are delayed speech development, and/or delay in development of social skills and in nonverbal communication. These children do not demonstrate regression in terms of loss of language or social skills.

Ethics statement. This study was approved by the Institutional Review Board (IRB) of the New York State Institute for Basic Research in Developmental Disabilities. The IRB reviewed this study in accordance with New York State Regulations and the HHS Office for Human Research Protections, including the "Human Subject Decision Chart 1," and found that *the research does not involve human subjects* because "the research does not involve intervention or interaction with the individuals", nor "is the information individually identifiable". The subjects cannot be identified, directly or through identifiers linked to the system, and the consent is not required.

Preparation of Brain Homogenates

The tissue samples were homogenized (10% w/v) in cold buffer containing 50 mM Tris-HCl (pH 7.4), 8.5% sucrose, 2 mM EDTA, 10 mM β-mercaptoethanol, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) at 4°C. For extraction of protein kinases, the homogenates were mixed with an equal volume of extraction buffer containing 40 mM Tris-HCl (pH 7.4), 300 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2% Triton, 5 mM sodium pyrophosphate, 2 mM β-glycerophosphate, 2 mM Na_3VO_4, 100 mM NaF, and 2 μg/ml leupeptin. The samples were allowed to stand on ice for 10 min, and then centrifuged at 135,000 g for 20 min at 4°C. The supernatants were collected, and the concentrations of total proteins in the supernatants were measured by the biocinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL).

Assay for PKA Activity

PKA activity was measured using the solid phase enzyme-linked immunosorbent assay (ELISA) kit from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA). In this assay, the substrate of PKA was pre-coated on the wells of a microplate. The microplate wells were soaked with 50 μl of kinase assay dilution buffer for 10 min. The buffer was then carefully aspirated from each well, and the brain samples were added to the appropriate wells. The kinase reaction was initiated by adding 10 μl ATP, and was carried out for 90 min at 30°C. It was terminated by emptying the contents of each well. A phosphosubstrate–specific antibody was added to the wells except in blank, and incubated for 60 min at room temperature, followed by washing 4 times with wash buffer. The peroxidase-conjugated secondary antibody was then added except in blank, and incubation was done for 60 min at room temperature. The wells were again washed 4 times with wash buffer. The color was developed with tetramethylbenzidine substrate, and it was proportional to the phosphotransferase activity of PKA. The reaction was stopped with acid-stop solution, and the absorbance was measured at 450 nm in a microplate reader. The absorbance was divided by the concentration of total protein (μg) in each sample, and the data are represented as relative PKA activity.

Western Blot Analysis

Total protein (15 μg) from brain homogenates of subjects with regressive- and non-regressive autism or control subjects was separated using a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose membrane. The membrane was blocked with Tris-buffered saline containing 5% fat-free dried milk for 2 h at room temperature, and further incubated overnight at 4°C with polyclonal antibody against C-subunit (isoform α) of PKA (Cell Signaling Technology Inc., Danvers, MA). The membrane was then washed 3 times with TBS-0.05% Tween 20, and incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The membrane was washed again, and the immunoreactive protein was visualized using enhanced chemiluminescent reagent.

Table 1. Case history and clinical characteristics of autism and control donors of brain tissue samples.

Brain tissue (UMB #)	Diagnosis	Autism Diagnostic tests	Age (y)	Sex	PMI (h)	Regressive autism	Other medical conditions	Medications	Cause of death
4671	Autism	ADIR, VABS, BSID-II	4.5	F	13	No			Multiple injuries from fall
1349	Autism	ADOS, VABS, BSID-II	5.6	M	39	Yes			Drowning
4849	Autism	ADIR, BSID-II, CARS	7.5	M	20	Yes	Lead poisoning		Drowning
1174	Autism	ADIR, VABS	7.8	F	14	No	Seizures	Depakote, Tegretol	Multiple-system organ failure
4231	Autism		8.8	M	12	No	Hyperactivity	Zyprexia, Reminyl	Drowning
797	Autism	ADIR	9.3	M	13	No	Attention deficit disorder, migraine headache	Desipramine	Drowning
1182	Autism	ADIR	10.0	F	24	Yes			Smoke inhalation
4899	Autism	ADIR	14.3	M	9	Yes	Seizures	Trileptal, Zoloft,Clonidine, Melatonin	Drowning
1638	Autism	ADIR	20.8	F	50	Yes	Seizures, Attention deficit hyperactivity disorder	Zoloft, Zyprexa, Mellaril, Depoprovera	Seizure-related
5027	Autism	WISC-R, Bender-Gestalt	38.0	M	26	No		Respirdal, Luvox	Obstruction of bowel
4670	Control		4.6	M	17				Commotio Cordis from an accident
1185	Control		4.7	M	17				Drowning
1500	Control		6.9	M	18				Motor vehicle accident
4898	Control		7.7	M	12		Hyperactive disorder	Concerta, Clonidone	Drowning
1708	Control		8.1	F	20				Motor vehicle accident
1706	Control		8.6	F	20		Congenital heart disease with heart transplant		Rejection of cardiac allograft transplantation
1407	Control		9.1	F	20		Asthma allergies	Albuterol, Zirtec, Alegra, Rodact, Flovent, Flonase	Asthma
4722	Control		14.5	M	16				Motor vehicle accident
1846	Control		20.6	F	9				Motor vehicle accident
4645	Control		39.2	M	12				Arteriosclerotic heart disease

ADI-R: Autism Diagnostic Interview Revised.
ADOS: Autism Diagnostic Observation Scale.
VABS: Vineland Adaptive Behavioral Scale.
BSID-II: Bayley Scales of Infant Development-Second Edition.
CARS: Childhood Autism Rating Scale.
WISC-R: Wechsler Intelligence Scale for Children-Revised.
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Table 2. Autism Diagnostic Interview-Revised test scores in donors of brain tissue samples.

Autism Diagnostic Interview-Revised (ADI-R)[a]							
Diagnostic Algorithm	**Cutoff score for autism**	**UMB 4671**	**UMB 4849**	**UMB 1174**	**UMB 797**	**UMB 4899**	**UMB 1638**
Impairments in reciprocal social interaction (Scores:0–30)	10	26	22	22	24	22	21
Abnormalities in communication:							
Verbal (Scores:0–26)	8	-	18	-	20	-	-
Non-verbal (Scores: 0–14)	7	13	N/A	11	13	14	11
Restricted, repeated and stereotyped behavior (Scores: 0–12)	3	3	8	6	6	8	7
Abnormalities of development evident at or before 36 months	1	5	3	5	-	4	5

a: Higher score represents greater impairment.
UMB 1182: ADI-R was conducted but the scores are not available. The donor met the criteria for a diagnosis of autism.
doi:10.1371/journal.pone.0023751.t002

Because PKA (C-α) and β-actin have similar molecular weights (42 KDa), polyclonal antibody against PKA (C-α) was stripped from nitrocellulose membrane, and the membrane was re-probed with monoclonal antibody against β-actin (loading control). The densities of all protein bands were measured by NIH Image J software, and the density of PKA (C-α) band was normalized with the density of β-actin for each sample.

Statistical Analysis

Initially, autistic and control cases were collected as age-matched pairs. As data for both members of a pair were not available in all cases, and data were approximately normally distributed, unpaired two-tailed t-tests were employed to make comparisons of PKA activity in various brain regions, and of overall PKA density between autistic vs. control cases. Comparisons among controls and autistic cases showing or not showing clinical signs of regression in function were made using one-way analysis of variance (ANOVA). To guard against type I error, a Bonferroni adjustment for multiple comparisons was made to the t-tests of multiple brain regions, and for the pairwise *post-hoc* t-tests comparing each pair of the three groups that were compared in the overall ANOVA. For purposes of this adjustment, tests of different hypotheses, i.e., of activity levels and of protein contents of PKA, were not considered to be multiple comparisons.

Results

PKA Activity in Different Brain Regions of Individuals with Autism and Age-Matched Control Subjects: Relationship with Regression in Autism

The activity of PKA was measured in the brain homogenates from the frontal, temporal, occipital, and parietal cortices, and the cerebellum in autistic and control subjects (Fig. 1). When all autism cases (regressive and non-regressive) were compared with the age-matched control group, no significant difference was found in PKA activity in any of these brain regions, although PKA activity in the frontal cortex was found to be reduced by 34.7% in the autism vs. control group. When the autism group was divided into two sub-groups (regressive and non-regressive), depending on whether there was a clinical history of regression or not, unadjusted two-tailed t-test showed a significant decrease in PKA activity in the frontal cortex of individuals with regressive autism as compared to the developmentally normal control group ($p = 0.0278$) and the non-regressive autism group

Table 3. Vineland Adaptive Behavioral Scales diagnostic test for autism in donors of brain tissue samples.

Vineland Adaptive Behavioral Scales (VABS)[a]							
	UMB 1349				**UMB 4671**		**UMB 1174**
	At age: 25 months		**At age: 33 months**		**At age: 39 months**		**At age: 6.4 y**
Domain (Scores:20–160)	**Standard Score**	**Age equivalent performance**	**Standard Score**	**Age equivalent performance**	**Standard Score**	**Age equivalent performance**	**Standard score**
Communication	57	9 months	69	18 months	52	10 months	41
Daily living skills	65	16 months	62	16 months	54	14 months	22
Socialization	60	9 months	71	17 months	51	4 months	52
Motor skills	-	-	-	-	65	24 months	-
Composite	-	-	-	-	51	13 months	35

a: Higher score represents better function.
According to the medical histories for UMB-4231 and UMB-5027, the donors had psychological evaluation, and met the criteria for a diagnosis of autism. Detailed information is not available.
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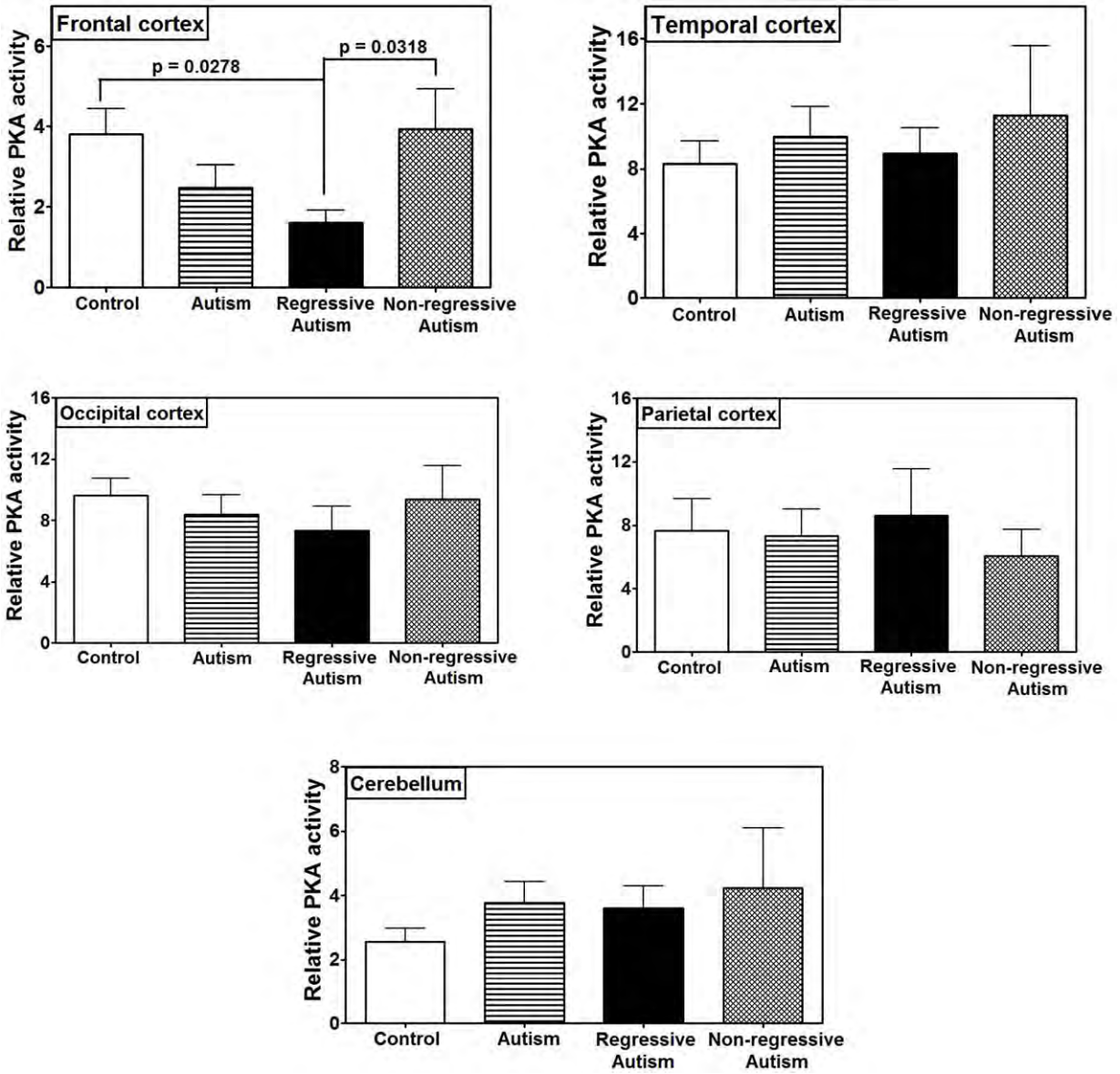

Figure 1. PKA activity in different brain regions from regressive autism, non-regressive autism, and age-matched control subjects. The autism group comprises combined regressive and non-regressive autism sub-groups. Brain homogenates were prepared, and activity of PKA was measured as described in Materials and Methods. Data represent mean ± S.E.
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($p = 0.0318$), but these differences did not remain significant after application of the adjustment for multiple comparisons. The mean ± S.E. of PKA activity in the frontal cortex was: 2.48 ± 0.57 in autism (regressive+non-regressive), 1.60 ± 0.31 in regressive autism, 3.94 ± 0.99 in non-regressive autism, and 3.80 ± 0.65 in control groups. The alteration in PKA activity was specific to the frontal cortex in regressive autism because it was not observed in other regions of the brain, i.e., the cerebellum and the temporal, parietal, and occipital cortices, suggesting that the changes observed in PKA activity were brain region–specific in regressive autism. PKA activity was also similar in all of the brain regions between non-regressive autism and control groups.

There was no significant difference in postmortem interval (PMI) between the autistic and control groups, or between the regressive autism and non-regressive autism groups. The mean ± S.E. of PMI was: 22.0 ± 4.2 in the autism groups (regressive+non-regressive, $n = 10$), 16.1 ± 1.22 in the control group ($n = 10$), 28.4 ± 7.2 in regressive autism ($n = 5$), and 15.6 ± 2.6 in the non-regressive autism group ($n = 5$). We also studied whether there was an inverse correlation between PMI and PKA activity. Correlation analysis between PMI and PKA activity for all autistic and control subjects did not reveal any such association (data not shown). Furthermore, the cerebellum and the temporal, parietal, and occipital cortices were not affected in subjects with regressive autism in comparison with control subjects, while the frontal cortex was affected in these individuals. These results suggest that PMI was not a contributing factor to the observed alteration in PKA activity in the frontal cortex of individuals with regressive autism. There was also no significant difference in age (mean ± S.E.) between the regressive autism (11.6 ± 2.7 years, $n = 5$) and non-regressive autism groups (13.7 ± 6.1 years, $n = 5$).

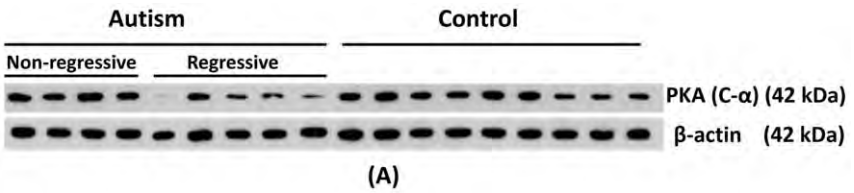
Protein Levels of Catalytic C-α Subunit of PKA in the Frontal Cortex of Individuals with Autism (Regressive and Non-Regressive) and Control Subjects

Because a decrease in PKA activity was observed in the frontal cortex of subjects with regressive autism as compared to control subjects and subjects with non-regressive autism, we analyzed whether the decreased activity of PKA is related to the reduced protein contents of PKA. The protein contents of the catalytic Cα unit of PKA were analyzed in the frontal cortex of individuals with autism (regressive and non-regressive) and age-matched controls by Western blotting (Fig. 2 A). The relative densities of the protein contents of PKA (C-α) normalized with β-actin are shown in Fig. 2 B. A one-way ANOVA comparing regressive and non-regressive autism cases and controls showed a significant difference in the protein contents among these three groups ($F_{[df=2,15]} = 9.770$, $p = 0.002$). *Post-hoc* pairwise comparisons among the groups revealed a significant decrease in the protein contents of PKA (C-α) in individuals with regressive autism (mean ± S.E = 0.34±0.09) as compared to control (mean ± S.E. = 0.64±0.05, $p = 0.019$, Bonferroni-adjusted) and individuals with non-regressive autism (mean ± S.E. = 0.83±0.09, $p = 0.002$, Bonferroni-adjusted), suggesting that the protein contents of PKA are affected in regressive autism. PKA contents were similar between non-regressive autism and control groups, and when the entire autism group (regressive and non-regressive) was compared with the control group.

Discussion

ASDs are complex neurodevelopmental disorders. The complexity of ASDs is further increased because some affected individuals fall in the sub-group of regressive autism [7]. Behavioral changes in regressive autism fall into two broad domains: (a) loss of vocalization and (b) loss of social skills. The rate of regressive autism varies from 15% to 62% of cases in different studies [4–7]. While Lord *et al.* reported that 29% of the children they studied who were diagnosed with autism had lost language skills for meaningful words, and another 9% lost non-word vocalizations [5], Goldberg *et al.* reported regression in 62% of children [4]. Loss of spoken words generally associates with loss of social behavior [6], but some affected children show only loss of social skills [4]. We report here that individuals with regressive autism have decreased PKA activity in the frontal cortex of the brain. This decreased PKA activity in autistic regression may be attributed to the decreased protein contents of PKA because the protein content of PKA (C-α subunit) was also decreased in the frontal cortex of individuals with regressive autism. Interestingly, such changes were not observed in other brain regions of individuals with regressive autism, or in the frontal cortex and other brain regions of individuals with non-regressive autism. These results suggest that alterations in PKA activity and PKA expression are specific to the frontal lobe in regressive autism.

Our results suggest that PMI and age cannot account for the observed alteration in PKA in regressive autism. Other factors, such as comorbidity with seizure disorder, reported for three of 10 autism cases (of which two had regressive autism, and one had non-regressive autism), and medications, reported for two regressive autism cases, four non-regressive autism cases, and two control cases, do not seem to be contributing factors to the altered activity or expression of PKA in regressive autism.



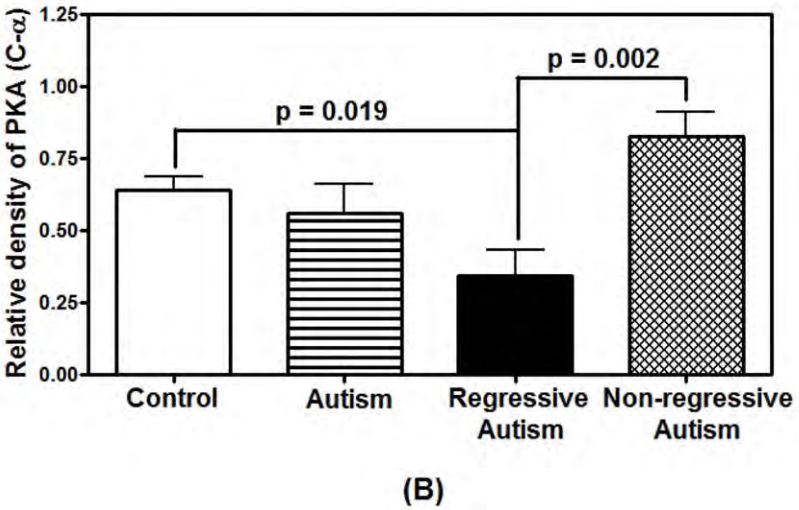


Figure 2. Relative protein levels of PKA (C-α) in the frontal cortex of regressive autism, non-regressive autism, and age-matched control subjects. Western blot analyses of C-α subunit of PKA in the frontal cortex of individuals with regressive and non-regressive autism, and age-matched control subjects are represented in Fig. 2A. The relative density of PKA (C-α) normalized with the density of β-actin (loading control) is shown in Fig. 2B. Data represent mean ± S.E.
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However, further studies with a larger autistic group should be done to explore this issue.

cAMP is one of the key factors for neuronal outgrowth, plasticity, and regeneration. Members of the cAMP-dependent second-messenger pathways participate in the regulation of cellular growth and differentiation and are also implicated in a variety of embryonic stages including brain development [24]. The PKA pathway is also recognized as an essential component in memory formation. Several studies in Drosophila have demonstrated the role of PKA in memory formation [25–29]. Mutations in the rutabaga gene, which encodes adenylate cyclase, caused significant defects in short-term memory [25]. Reduced expression or activity of DC0 (the gene encoding the catalytic subunit of PKA) caused deficits in learning, short-term memory, and middle-term memory [26–28]. Studies have also shown that pharmacological agents such as cAMP analogs and rolipram (an inhibitor of PDE), which are known to increase PKA activity, could improve memory [30,31].

G-protein–coupled adenylate cyclase converts ATP to cAMP, which in turn binds to regulatory subunits of PKA. Following this event, catalytic subunits of PKA are released, which are the activated forms of PKA. PKA then phosphorylates and alters the activity of enzymes and many target proteins such as ion channels, chromosomal proteins, and transcription factors. cAMP response-binding protein (CREB) is one of the targets of PKA-mediated phosphorylation. CREB, upon activation by PKA, binds to certain DNA sequences (cAMP response elements), thereby stimulating the transcription of downstream genes and the synthesis of proteins. The CREB transcription factor is also required for long-term memory formation [32–34]. It is possible that a decrease in the activity of PKA in regressive autism may result in reduced phosphorylation of CREB, and thus reduced transcription and altered synthesis of some proteins.

Given that PKA is activated by cAMP, and PDE regulates the levels of cAMP, a discussion on PDE becomes imperative. Altered levels of PDE4 in the cerebella of autism subjects were reported by Fatemi and group [23]. Other studies have suggested a role of PDE4 in learning and memory in behavioral models of mice, rats, and monkeys [35,36]. PDE4 is also reported to be involved in behavior sensitivity to antidepressant drugs in animals [37]. PDE inhibitors such as rolipram could improve object recognition [38,39], passive avoidance [40,41], radial arm maze [40–42], Morris water maze [43], and contexual fear conditioning [30,43,44]. PDE4 has also been studied as a potential therapeutic target for depressive disorders. It has been suggested that rolipram may have potential therapeutic benefits for major depression [45], Alzheimer's disease [36,46], Parkinson's disease [47,48], schizophrenia [49,50], and tardive dyskinesia [51,52].

Several reports suggest that some proteins related to the PKA pathway are involved in autism. Extensive evidence indicates hyperserotonemia in autism [53–55]. PKA regulates serotonergic activity in the brain [56]. Galter and Unsicker [57] reported that co-activation of cAMP- and tyrosine receptor kinase B (TrkB)–dependent signaling pathways plays an important role in maintaining the serotonergic neuronal phenotype. TrkB is also regulated by the cAMP/CREB pathway in neurons [58]. Furthermore, transcriptional activity of the engrailed-2 gene is also regulated by PKA [59]. The importance of engrailed can be envisioned because of its crucial roles in brain development [60] and in the development of autism [61–65].

In conclusion, this study suggests that the frontal cortex may be the region of the brain involved in regressive autism, where abnormalities such as decreased activity and expression of PKA can affect the signal transduction. It may have multiple effects on signal transduction pathways, which may also influence serotonergic neurons, TrkB, and engrailed-2, all of which have been suggested to be involved in the development of autism.

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Author Contributions

Conceived and designed the experiments: AC. Performed the experiments: LJ. Analyzed the data: AC VC MJF. Contributed reagents/materials/analysis tools: AC. Wrote the paper: AC VC.

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